REMARKS

Claim Amendments

Claims 17, 19-21, 23-24, 28-29, 31, 33-34, 38-39, 41, 43, 46, 47, 49 and 54-56 are currently pending in the application. Claim 3-6, 9-16, 18, 22, 25-26, 27, 30, and 35-37 were previously cancelled. Claims 1-2, 7-8, 32, 40, 42, 44-45, 47-48, 50-53 have been newly cancelled without prejudice to pursuing subject matter of these claims in this or another application. Claims 17, 19-21, 23-24, 28-29, 31, 33-34, 38-39, 41, 43, 46, 47, 49, and 54-55 are newly amended; while claim 56 is newly added. The claim amendments find support in the specification and are discussed below.

Independent claims 17, 19, 54 and 55 have been amended to ensure clarity within the claims. Newly added claim 56 notes that quantification of the level of amplification products quantified in step (b) can be effected by determining a quantity of the amplification product relative to a housekeeping gene. Support for this amendment is found in paragraph [0163] and [0164] and in Figure 5B of the published application US 2007/0031841 (hereinafter the "Published Application").

Claim Objections

The Examiner has objected to the following informalities:

(a) Claim 1 read "diagnosing or prognosing a disease a test subject". As noted above independent claim 1 has been cancelled.

(b) Claim 17 recited "of blood samples which have 'no' been fractionated". The word 'no' replaced with the proper term 'not'.

(c) Claim 21 recited "an amplification products". Claim 21 has been amended to recite "amplification products" and the term 'an' deleted.

(d) Claim 34 was objected to under 37 CFR 1.75(c) as being of improper dependent form. As noted above, claim 1 has been cancelled and claim 34 has been amended to delete the dependency of claim 34 from claim 1.

In view of the above amendments, the Applicant believes that they have overcome the informalities objections raised by the Examiner. The Applicant respectfully requests

reconsideration and withdrawal of the rejection.

35 U.S.C. § 112, 2nd Paragraph Rejections - Indefiniteness

The Examiner has objected to claim 17, and those claims dependent from claim 17, as being indefinite because claim 17 recites that each of said genes is identified "as a further marker of said disease" yet no marker was identified as a first marker within the claim. Claim 17 has been amended to delete the reference to a "further" marker. The Applicant has thus clarified that the method of claim 17 identifies two or more markers useful for diagnosing disease. In view of the above amendments, the Applicant believes that they have overcome the indefiniteness rejection raised by the Examiner against claim 17 and those claims dependent upon claim 17. The Applicant respectfully requests reconsideration and withdrawal of the rejection.

35 U.S.C. § 112, 1st Paragraph Rejection – Written Description

The Examiner has objected to claims 39 and 46 as failing to comply with the written description requirement. More particularly the Examiner has objected to the language "subjects having said disease...have no overt symptoms with respect to said disease" found in claim 39, and the language "said subjects having said disease are asymptomatic with respect to said disease" found in claim 46. The Examiner also argues that the phrase "subjects having said disease are asymptomatic with respect to said disease" is new matter.

To satisfy the written description requirement, patents must describe the technology that is sought to be patented so as "to satisfy the inventor's obligation to disclose the technologic knowledge upon which the patent is based, and to demonstrate that the patentee was in possession of the invention that is claimed." Capon v. Eshhar, 418 F.3d 1349, 1357, 76 USPQ2d 1078, 1084 (Fed. Cir. 2005). The inquiry is primarily factual and depends on the nature of the invention and the amount of knowledge imparted to those skilled in the art by the disclosure (In re Wertheim, 541 F.2d at 262, 191 USPQ at 96).

The Examiner agrees that the Applicant has reduced to practice an example whereby the insulin gene was identified as a biomarker which is differentially expressed between control patients and a person having asymptomatic diabetes (paragraph [0164] and Figure 5A of the

Published Application). The Applicant also notes the additional teaching of the second gene *zfp*, which is also identified by the Applicant as differentially expressed between control patients and persons having asymptomatic diabetes (See paragraph [0162] and [0164] and Figure 5B of the Published Application). Therefore, the Applicant has taught a method of identifying two or more markers useful for diagnosing asymptomatic diabetes, using primers specific for the insulin and *zfp* genes, respectively, producing amplification products from RNA of blood samples which have not been fractionated into cell types from subjects having asymptomatic diabetes, quantifying a level of said amplification products (see Figure 5A and Figure 5B), and quantifying a level of the control amplification products (see Figure 5A and Figure 5B) to determine a difference of expression indicative of asymptomatic diabetes.

The Examiner however is concerned that there is insufficient written description support because "the specification does not provide any discussion of **how the disclosed methods could be applied to any patient asymptomatic of disease**" (p.5 of Office Action dated March 8, 2007 hereinafter the "Office Action"). The Examiner seems, in particular, to be concerned that the specification does not disclose how to identify patients who are asymptomatic for disease. For example, the Examiner suggests that identifying patients who are asymptomatic for disease is not possible - stating "the use of the term "asymptomatic diabetes" is somewhat of a misnomer because in order to in fact determine a patient as being within this class, some symptoms of diabetes must be present" (p.5 of Office Action), and "it is not even clear that one could HAVE heart failure but be asymptomatic for heart failure" (p.6 of Office Action). Thus the Examiner suggests that the presence of disease cannot be determined unless the patient has symptoms. This is not correct, and does not take into account the meaning of the term "symptom" as would be understood by a person skilled in the art. A symptom of disease is defined as something abnormal that is subjectively experienced by a patient. See, for example, the definition of "symptom" in Stedman's 27th edition Medical Dictionary (enclosed) which defines a symptom as "any morbid phenomenon or departure from the normal in structure, function, or sensation, **experienced by the patient** and indicative of disease" (p.1742). In contrast, "signs" of disease are defined as "any abnormality indicative of disease, discoverable on examination of the patient; an objective indication of disease, in contrast to a symptom, which is a subjective indication of disease" (Stedman's 27th edition Medical Dictionary p.1635). Diagnosing disease in asymptomatic patients using objective medical tests was well known in the art for many diseases prior to the Applicant's invention.

For example, glycemic testing to screen asymptomatic patients for Type II diabetes was well known before the invention date, as were recommendations to screen for asymptomatic patients based on these tests as set out by the American Diabetes Association (see for Example Diabetes Care January 1999 Vol. 22 p. S20 enclosed). Also, the use of tests such as transthoracic two-dimensional echocardiography (TTE) to screen for congenital heart defects which result in sudden death was often performed in athletes prior to the Applicant's invention (see, for example, "In vivo detection of coronary artery anomalies in asymptomatic athletes by echocardiographic screening." Chest 1998 July 114(1) 89-93 (enclosed)). Identification of asymptomatic patients in other disease areas was also well known before the date of the Applicant's invention. For example, screening tests such as fecal occult blood test, digital rectal examination, and endoscopy to detect the presence of colorectal cancer have been well documented since as early as 1986 (Arch Surg. 1986 Nov;121(11):1347-52 (enclosed)) and recommended by many institutions including the American Cancer Society (see Byers T, Levin B, Rothenberger D, Dodd GD, Smith RA. American Cancer Society guidelines for screening and surveillance for early detection of colorectal polyps and cancer: update 1997. American Cancer Society Detection and Treatment Advisory Group on Colorectal Cancer CA Cancer J Clin. 1997;47:154-60 (enclosed)). Also, the use of a pap smear for early detection of cervical cancer (see Canadian Medical Association Journal (1982) 127(7) "Cervical cancer screening programs, summary of the 1982 Canadian task force report (enclosed)) is well documented, and blood tests to screen for hyperthyroidism to diagnose asymptomatic patients was performed as early as 1862. (see Abstract Presse Med. 1983 Jan 22;12(3):147-51 (below)).

There is nothing in law which suggests that reduction to practice of a single species encompassed within a genus is insufficient to support written description (Enzo Biochem, 323 F.3d at 966, 63 USPQ2d at 1615; Noelle v. Lederman, 355 F.3d 1343, 1350, 69 USPQ2d 1508, 1514 (Fed. Cir. 2004) (Fed. Cir. 2004)). Furthermore, it is well known that a patent application need not teach, and preferably omits, that which was well known in the art (Hybritech v. Monoclonal Antibodies Inc. 802 F2d. 1367 (1986)). The Applicant describes the problem faced in the prior art very broadly, noting the prior art was deficient in "non-invasive methods to screen for tissue specific disease" (see paragraph [009] of the Published Application) and further describes the invention broadly, noting "It is believed that the present finding can potentially revolutionize the way diseases are **detected**, diagnosed, and monitored... because it provides...**quick screening** for tissue specific transcripts...**as they reflect disturbances in the**

homeostasis in the human body" (see paragraph [0013] of the Published Application). Thus the Applicant's invention is not limited to identifying transcripts which diagnose disease only once it has progressed so as to become symptomatic, but recognizes that the blood can detect all disturbances to the status quo. The fact that a specific example has been reduced to practice whereby two markers are identified which are useful to screen for asymptomatic diabetes provides further recognition within the specification of the broad applicability of the disclosed method to patients who are asymptomatic for disease in the identification of disease markers. As described above, the identification of patients who are asymptomatic for disease was well known in the art at the time of the Applicant's invention and as such, the use of these patients within the methods disclosed by the Applicant would be well understood. In view of the above arguments, the Applicant respectfully requests reconsideration and withdrawal of the rejection.

35 U.S.C. § 112, 1st Paragraph Rejection – Enablement

Claims 1, 2, 32, 44, 45, 47 and 48 are rejected for failing to comply with the enablement requirement. Furthermore claims 20, 21, 23, 24, 28, 29, 31, 33, 34, 38, 41 and 43 are also rejected for failing to comply with the enablement requirement, to the extent that these claims are dependent on independent claims 1 and 2. More specifically the Examiner contends that the "clear language of the claim is that the determination of a difference in step (c) results in diagnosing or prognosing a disease in a test subject", and as such the Applicant is required to disclose "minimal sets of two or more genes whose differential expression between a sample and a control is sufficient to determine that a disease is present or to predict the outcome of disease".

While disagreeing with this view as to the requirement to enable independent claims 1 and 2, and those claims dependent thereon, the Applicant has nonetheless cancelled independent claims 1 and 2 subject to the Applicant's rights to pursue the subject matter of these claims in another application. In view of the above amendments, the Applicant respectfully requests reconsideration and withdrawal of the rejection.

35 U.S.C. § 112, 1st Paragraph Rejection – Written Description

The Examiner has rejected claim 43 under 35 U.S.C. 112 1st paragraph as failing to comply with the written description requirement. More specifically the Examiner has objected to

these claims because they are drawn broadly to the "diagnosis or prognosis of colorectal cancer, diabetes or heart failure by the detection of expression of two or more genes".

The Applicant has amended claim 43 so that it no longer depends from claims 1 and 2. As such, newly amended claim 43 is no longer drawn to methods which require the "diagnosis or prognosis" of the selected group of diseases. As such, in view of the above amendments, the Applicant respectfully requests reconsideration and withdrawal of the rejection.

35 U.S.C. § 103 – Ringel and Kinoshita

The Examiner has rejected claims 7, 8, 20, 21, 23, 24, 28, 29, 31, 33, 34, 38, 40, 42, 49, 52, and 53 as being obvious over the combination of Ringel et al. in view of Kinoshita et al. The Applicant disagrees that Ringel et al. in view of Kinoshita et al. makes the above noted claims obvious; nevertheless, in the interest of expediting prosecution, and subject to the Applicant's rights to pursue the subject matter of these claims in another application, the Applicant has cancelled independent claims 7, 8, 52 and 53. Furthermore the Applicant has amended dependent claims 20, 21, 23, 24, 28, 29, 31, 33, 34, 38, 40, 42, and 49 so as to remove reference to cancelled claims 7 and/or 8 as appropriate. In view of the above amendments, the Applicant respectfully requests reconsideration and withdrawal of the rejection.

35 U.S.C. § 103 – Nagai and Kephart

The Examiner has rejected claims 7, 8, 20, 21, 23, 24, 28, 29, 31, 33, 34, 38, 40, 42, 52, and 53 as being obvious over the combination of Nagai et al. in view of Kephart. The Applicant disagrees that Nagai et al. in view of Kephart makes the above noted claims obvious; nevertheless, in the interest of expediting prosecution, and subject to the Applicant's rights to pursue the subject matter of these claims in another application, the Applicant has cancelled independent claims 7, 8, 52 and 53. Furthermore the Applicant has amended dependent claims 20, 21, 23, 24, 28, 29, 31, 33, 34, 38, 40, 42, and 49 so as to remove reference to cancelled claims 7 and/or 8 as appropriate. In view of the above amendments, the Applicant respectfully requests reconsideration and withdrawal of the rejection.

35 U.S.C. § 103 – Wong and Kephart

The Examiner has rejected claims 1, 2, 7, 8, 17, 19, 20, 21, 23, 24, 28, 29, 31, 33, 34, 38, 40, 42, 44, 45, 47, 48, 50, 51, 52, 53 and 55 as being obvious over the combination of Wong et al. in view of Kephart.

As described in detail below, the combination of Wong et al. and Kephart fails to make the invention as claimed obvious. First, there is no prima facie case because Kephart does not fairly suggest to a person skilled in the art that RNA expression levels in the blood samples claimed are the equivalent of expression levels in PMNCs. Post-filing reference Du et al.[1] is provided to demonstrate that expression within each cell type differs and expression in polymorphonuclear cells in particular cannot merely be considered background. In addition, Kephart teaches away from the combination by teaching blood cells provide unwanted background and thus methods which increase the potential background as taught by Kephart are to be avoided.

Second, there is no reasonable expectation of success in detecting *afp* encoded RNA in healthy individuals using the methods taught by Kephart. Wong et al. itself does not enable detection and quantification of *afp* encoded RNA in healthy individuals and each of pre-filing references Matsumura et al., 1994[2], Matsumura et al., 1995[3], Funaki et al.[4] Lemoine et al.[5] and Liu et al.[6] demonstrate that *afp* RNA was not detected in healthy individuals using methods akin to Kephart. Finally, post-filing reference Lupberger et al.[7] provides additional evidence that the detection of *afp* RNA in healthy individuals would not be enabled by the methods of Kephart as applied to Wong et al. because the volumes required by Kephart's techniques to detect *afp* RNA in healthy individuals are overwhelmingly large. In addition, Kephart teaches away from using

[1] Du et al. (2006) (Genomic profiles for human peripheral blood T cells, B cells, natural killer cells, monocytes, and polymorphonuclear cells: Comparisons to ischemic stroke, migraine and Tourette syndrome) Genomics 87 693-703)

[2] Matsumura et al., 1994 Detection of alpha-fetoprotein mRNA, an indicator of hematogenous spreading hepatocellular carcinoma, in the circulation: a possible predictor of metastatic hepatocellular carcinoma. Hepatology. 20:1418

[3] Matsumura et al., 1995 Sensitive assay for detection of hepatocellular carcinoma associated gene transcription (alpha-fetoprotein mRNA) in blood. Biochem Biophys Res Commun. 207:813

[4] Funaki et al., 1994 (Highly-sensitive identification of alpha-fetoprotein mRNA in circulating peripheral blood of hepatocellular carcinoma patients. Life Sci. 57:1621)

[5] Lemoine et al. 1997 Prospective evaluation of circulating hepatocytes by alpha-fetoprotein mRNA in humans during liver surgery. Ann Surg. 226:43)

[6] Liu et al., 1998 The detection of circulating hepatocellular carcinoma cells in peripheral venous blood by reverse transcription-polymerase chain reaction and its clinical significance. Zhonghua Wai Ke Za Zhi. 36:60.

[7] Lupberger J, Kreuzer KA, Baskaynak G, Peters UR, le Coutre P, Schmidt CA. Links
Quantitative analysis of beta-actin, beta-2-microglobulin and porphobilinogen deaminase mRNA and their comparison as control transcripts for RT-PCR. Mol Cell Probes. 2002 Feb;16(1):25-30.

rare mRNAs and Lupberger et al. and pre-filing reference Matsumura et al., 1994 provide evidence that *afp* encoded RNA in healthy individuals is a rare mRNA in comparison with beta-actin.

A. No Prima Facie Case – Prior Art Does Not Teach All Elements of the Claimed Invention

(i) Wong does not teach quantifying the amount of RNA expressed by afp and alb in <u>all RNA expressing cells in whole blood</u> and <u>determining a difference</u> as required by the claims and Kephart does not provide these missing elements

The prior art reference (or references when combined) must teach or suggest **all the claim limitations** (In re Royka, 490 F.2d 981, 180 USPQ 580 (CCPA 1974)).

While the Examiner recognizes that Wong et al. "do[es] not teach <u>detection</u> of gene expression in a blood sample which has not been fractionated into cell types" (p.17 of Office Action), the Applicant notes that, in fact, Wong does not teach ANY of the following limitations as required pursuant to the Applicant's claims:

(i) **detecting** *alb* and *afp* gene expression <u>in a blood sample which has not been fractionated into cell types (ie peripheral mononuclear cells, polymorphonuclear cells etc.)</u> in subjects having disease;

(ii) **quantitating the amount** of *alb* and *afp* gene expression <u>in a blood sample which has not been fractionated into cell types</u>; and

(iii) **determining a difference** between the amount of *alb* and *afp* gene expression in the blood samples of the subjects having disease and comparing with a quantified amount of *alb* and *afp* gene expression <u>in blood samples which have not been fractionated into cell types</u> in control subjects **such that the difference identifies *alb* and *afp* as markers of said disease.** Similarly, Wong et al. does not teach detecting, quantifying, and determining a difference in levels of *afp* or *alb* RNA such that the differences identify *alb* and *afp* as markers of disease using <u>RNA of unfractionated cells of lysed blood samples</u> as between subjects having disease and control subjects.

The Examiner notes that Wong et al. detects *afp* and *alb* encoded RNA only in a Ficoll-fractionated preparation of peripheral mononuclear cells (PMNCs). Wong et al. does not teach

the amount of *alb* and *afp* gene expression in samples which include polymorphonuclear cells, does not quantitate the amount of *alb* and *afp* gene expression in samples which includes polymorphonuclear cells in individuals having disease, and does not determine a difference in the amount of *alb* and *afp* RNA quantitated in subjects having disease, as compared with control samples, when the quantified amount of *alb* and *afp* in control samples includes the amount of *alb* and *afp* RNA detected in polymorphonuclear cells.

The Examiner suggests, in the context of the s.103 combination of Nagai and Kephart, that the missing elements described in the preceding two paragraphs are provided by Kephart because Kephart "clearly considered that the results would be similar enough that their method be used instead of methods which require the isolation of mononuclear cells" (p.28 of Office Action), and teaches that "the fact that there are additional cell types in the blood sample which express genes may [merely] add to the backgrounds of the assay" (p. 28 of Office Action).

With respect, this interpretation of Kephart goes beyond what would reasonably be understood by a person skilled in the art. While Kephart discusses the technique of RT-PCR broadly stating "RT-PCR is an extremely sensitive and versatile technique that can be utilized for a variety of applications. In this technique, total or selected RNA [i.e., poly(A)+] is used as a substrate for detecting specific transcripts, to estimate relative expression levels of a gene of interest or to amplify cDNA products for use in applications such as cloning or *in vitro* translation" (p.11), a person skilled in the art would not understand this to mean the Kephart methods will be successful in all instances where RT-PCR and blood RNA are utilized. The Applicant notes that "a reference must be considered not only for what it expressly teaches, but also for what it fairly suggests" (In re Burckel, 592 F.2d 1175, 1179, 201 USPQ 67, 70 (CCPA 1979)). The Applicant further notes that a prior art reference's expansive predictions about its own significance do not necessarily govern (In re Bell, 991 F.2d 781, 784 (C.A.Fed.1993)).

In this case, however, Kephart is not making overly expansive predictions, but merely states that the **utility of the application depends upon the researcher's needs** (ie "Whole blood is a valuable tissue source for RT-PCR methodologies used in a variety of research applications. ...The three protocols, which differ in their basic strategy, enable researchers to utilize the methods that **best suit their laboratory and research needs**" (p.14)) and "the extreme simplicity and high throughput capacity of this technique may make it an appropriate alternative to traditional RNA isolation techniques **for use in some RT-PCR applications"** (p.12 last sentence).

Kephart notes other limitations of his techniques as well, stating "increasing amounts of cell derived inhibitory agents at higher concentrations (Figure 3, lane 2)" (see also Figure 2, lane 2) and the potential for increased background due to contamination may interfere with the detection of rare RNAs". To the extent that Kephart can even be considered to look at the question of quantification as compared to mere detection, Kephart demonstrates that amplification is not linear within the small volumes taught (ie less than 10μl). There is no evidence that Kephart is suggesting that quantification of the amount of RNA will not be affected by the additional cell types found in whole blood samples and that these additional cells merely add background noise when determining a difference relative to hepatocellular carcinoma. If Kephart was suggesting that quantification of levels of RNA are comparable between whole blood samples and fractionated blood samples, a person skilled in the art would at least expect Kephart to provide data to compare the level of the housekeeping gene beta-actin detected in whole blood samples against the levels of beta-actin isolated from mononuclear cells. No such data is presented.

Clearly, therefore, Kephart **cannot even fairly suggest** that quantification of the amount of RNA expressed by both ***alb* and *afp*** is not affected by the additional cell types found in whole blood samples such that these additional cells merely add background noise and will not affect a determination of a difference between the levels of *alb* and *afp* RNA as between individuals having HCC and control subjects.

Post filing reference Du et al. provides evidence which demonstrates that each of the blood cell types can provides its own unique contribution towards a measured level of expression as between disease and control subjects. Du et al. states "**several blood RNA isolation methods have been used to date...however, the RNA isolated using these methods comes from various blood cell subsets that originate from different developmental lineages, perform separate and distinct biological functions, and, most likely, have very different genomic expression signatures."... "a disease may predominately affect one specific blood cell subtype while sparing others. Therefore, characterizing the contribution of every blood cell subtype to the overall blood genomic pattern may be essential to distinguish significant genomic changes from noise, interpret the disease-related patterns, and decide on the proper blood cell types to perform follow-up confirmatory analyses**" (see p.693 last paragraph and p.694 1st paragraph). In fact, the experiments in Du et al. indicate that certain cell types contribute more to certain diseases than others noting "Tourette syndrome, ...appears to be

associated with changes in gene expression in NK cells and/or CD8+ cells ..."[while]blood genomic response following acute ischemic stroke was predominantly from PMNs [polymorphonuclear cells] based upon the current finding and those of our recent ischemic stroke study...[whereas] blood genomic patterns for migraine headache is more heterogeneous, with several blood cell types affected, including platelets and monocytes" (see p.701, 1st column). Thus Du et al. provides additional support to demonstrate that Kephart combined with Wong et al., do not teach each of the elements of the claimed invention. In view of the above arguments, the Applicant respectfully requests reconsideration and withdrawal of the rejection.

(ii) Wong et al. <u>teaches away</u> from the combination by teaching a determination of the amount of RNA expressed in HCC ONLY because of the difficulty with background already found in PMNCs alone.

The prior art reference (or references when combined) must teach or suggest all the claim limitations (In re Royka, 490 F.2d 981, 180 USPQ 580 (CCPA 1974)). A prior art reference must be considered in its entirety, i.e., as a whole, including portions that would lead away from the claimed invention. W.L. Gore & Associates, Inc. v. Garlock, Inc., 721 F.2d 1540, 220 USPQ 303 (Fed. Cir. 1983), cert. denied, 469 U.S. 851 (1984).

In this case, the Examiner has suggested that each of the elements of the claims is taught by Wong et al., with the exception that Wong et al. "does not teach detection of gene expression in a blood sample which has not been fractionated into cell types" and that Kephart provides this missing element. As stated above, Wong et al., in fact, does not teach ANY of detecting, quantifying, and determining a difference in expression levels of *afp* or *alb* RNA such that the differences identify *alb* and *afp* as markers of disease using either (i) a blood sample which has not been fractionated into cell types, or (ii) RNA of unfractionated cells of lysed blood samples as between subjects having disease and control subjects.

Wong et al., however, does specify that the purpose of their teachings is to "**develop a semi-quantitive estimation of the amount of "<u>hepatocyte specific" mRNAs</u> in the circulation of normal subjects and HCC patients <u>for differential detection of HCC cells rather than normal PMNCs</u>** (see Introduction pg. 628). Wong et al. notes that while the expression of *afp* and *alb* genes had previously been associated with the presence of metastasis (e.g. see introduction citing Hillaire et al, 1994, Matsumura et al., 1994, Kar and Carr, 1995; Komeda et al., 1995 and Nambu et al. 1995), the specificity of the genes were brought into

question because *alb* and *afp* were detected in the peripheral blood of patients that did not have hepatocellular carcinoma. Thus the fact that Wong et al. teaches "quantitative estimation of the amounts of "**hepatocyte-specific" mRNAs for differential detection of HCC cells rather than normal PMNCs"** is critical. This **teaches away** from detecting and quantifying the contribution of even peripheral blood mononuclear cells in determining a difference indicative of disease. Thus given the teachings of Wong et al. that even PMNCs are contributing unwanted background due to "illegitimate transcription" and the importance of quantitating so as to ensure that the unwanted background is removed from the calculation of the detection of circulating HCC cells, a person skilled in the art would be led away from the combination with Kephart since this combination would utilize a method which has the potential to introduce even more unwanted background by introducing more blood cells. In view of the above arguments, the Applicant respectfully requests reconsideration and withdrawal of the rejection.

B. No Prima Facie Case – No Reasonable Expectation of Success

According to the Examiner, Wong et al. teaches all of the elements of the claims with the exception that "Wong et al. does not teach detection of gene expression in a blood sample which has not been fractionated into cell types".

Even if, contrary to the Applicant's arguments above, the Examiner considers that Wong et al. teaches all of the elements of the current claims, in order for the combination of Wong et al. and Kephart to result in a prima facie case, there must be a reasonable expectation of success that the combination will result in the performance of a method falling within the scope of the Applicant's claims (In re Royka, 490 F.2d 981, 180 USPQ 580 (CCPA 1974) In re Rinehart, 531 F.2d 1048, 189 USPQ 143 (CCPA 1976)). Thus, the practicing of the invention as taught by Wong et al., but applying one of the methods taught by Kephart, must reasonably be expected to resut in: (i) **detecting the presence of RNA encoded by *afp* and *alb* in a sample from each of one or more subjects having HCC, and** (ii) determining a difference as compared to a **quantified amount of RNA encoded by *afp* and *alb* having been detected in samples from healthy control subjects** in a sample as required by claims 17, 19, 54 and 55. Thus, the combination of Kephart and Wong et al. must result in a reasonable expectation of success of the detection of both *afp* and *alb* in healthy control patients and a reasonable expectation of success that there can be a quantifiable amount of RNA encoded by both *afp* and *alb* RNA in healthy control patients.

(i) According to the self described limits of their own assay, Wong et al. does not enable detection of afp in healthy individuals so as to obtain a quantified amount; therefore, there can be no reasonable expectation of success when using RNA from whole blood in place of RNA from PMNCs.

Wong et al. contends at p. 631, 1st column, to have achieved detection of *afp* RNA in PMNCs of 2 out of 18 normal subjects tested, and discloses, with reference to Fig. 4, that <u>none of the samples from normal subjects exhibited *afp* mRNA levels exceeding those corresponding to 10 pg of HepG2 total RNA;</u> however, they also teach at p. 631, 1st paragraph, that "<u>levels of *afp* mRNA corresponding to 10 pg of HepG2 total RNA are below the limit of detection"</u> of their own assay.

Furthermore, the Applicant's claims require not just that the RNA be detected in the control samples, but that comparison be made with **a quantified level of control RNA** encoded by said gene. Wong et al. provides at Fig. 2, RT-PCR calibration curve data showing levels of *afp*-specific amplimer corresponding to HepG2 total RNA within a range starting from a minimum of 165 pg of HepG2 total RNA. This is in direct contrast to the disclosure at Fig. 1, right panel, of similar calibration data for *alb* RNA disclosing the entire lower end of the range of detection, as well as background levels in the absence of HepG2 cells in normal subjects. Wong *et al.* fails to disclose the results of *afp* RNA detection corresponding to levels below 165 pg HepG2 total RNA, as well as background levels in the absence of HepG2 cells observed in normal subjects. Thus, the kinetics of, and hence the significance and utility for quantitation of *afp* RNA levels below those corresponding to 165 pg HepG2 total RNA taught by Wong *et al.* cannot be ascertained.

In fact, the detection Wong et al. claims to achieve for the two normal subjects appears to represent non specific amplification. For example, even in the case of *alb*, for which background levels in the absence of HepG2 cells are disclosed, Wong *et al.* fails to provide any data enabling differentiation of target gene-specific amplification from background levels in the absence of HepG2 cells.

Given the fact that Wong et al. themselves teach that the level of *afp* RNA is well below their own ability to detect and quantify using PMNCs, there can be no reasonable expectation of success when using methods which do not utilize fractions of white blood cells as taught by Kephart. As such, the combination of Wong et al. with Kephart does not render the claimed

invention obvious.

(ii)The prior art demonstrates that the use of Kephart methods DID NOT RESULT in detection of afp RNA in healthy individuals even using RT-PCR techniques far more sensitive than that employed by Wong et al – therefore there is no reasonable expectation of success with respect to the combination.

The Examiner asserts that Wong *et al.* teaches all of the limitations of the Applicant's claims, with the exception that "Wong et al detect[s] RNA in peripheral mononuclear cells". The Examiner further suggests that "it would have been prima facie obvious to one of ordinary skill in the art at the time that the invention was made to have modified the methods taught by Wong et al. "**so as to have avoided the step of isolating lymphocytes** using a method as taught by Kephart". The Examiner states that "Kephart et al. clearly considered that the results would be similar enough when using methods which avoided the step of isolating lymphocytes" (p.28 Office Action) and thus "one would have been motivated to modify the methods taught by Wong et al. as taught by Kephart in order to avoid the need to isolate the blood cells, and to utilize the systems taught by Kephart" (p.17 of the Office Action). Clearly all of the methods taught by Kephart are slightly different from each other, however they all isolate RNA from whole blood without requiring the time consuming step of isolating a fraction of leukocytes. Thus according to the Examiner, Kephart teaches methods which detect and measure RNA in whole blood that produce results which are equivalent to methods which measure the level of RNA in only PMNCs and to the extent that the Kephart methods are different than those described in Wong et al., they would merely add to the background of detection.

The Applicant respectfully submits that claims drawn to two or more genes require detection of RNA encoded by each of the two or more genes in the control samples; therefore, there must be a reasonable expectation of success that Wong et al. when combined with Kephart will result in detection of *afp* RNA in the healthy control samples. The prior art, however, is replete with examples which demonstrate that methods which avoid the step of isolating lymphocytes and attempt to detect *afp* expression in **whole blood** are **unable to detect *afp* RNA in healthy individuals**. As such, there would be no reasonable expectation of success by a person skilled in the art that the methods taught by Kephart to avoid isolating lymphocytes when combined with Wong et al. would result in detection of *afp* RNA in healthy individuals so as to

make the methods as encompassed by the Applicant's claims obvious.

All of the following references utilize methods to isolate RNA which result in isolation of RNA from **whole blood** and avoid the use of a fractionated sample of leukocytes (ie namely PMNCs) as taught by Wong et al. In addition, each of the following references utilize a method of detecting *afp* RNA which is, in one case essentially at least as sensitive, and in all other cases, significantly more sensitive than the method utilized by Wong et al. Despite this, none of the references noted below are able to detect *afp* RNA in healthy individuals as required by the claims of the Applicant.

Matsumura et al. 1995 teaches isolation of all nucleated cells of peripheral blood by first lysing the red blood cells, (p. 814, under "MATERIALS AND METHODS") and is similar to the most sensitive (freeze-thaw based) method taught by Kephart[8] which lyses red blood cells using a rapid freeze thaw and in which "mRNA containing leukocytes are collected by centrifugation" (p. 12). Matsumura et al., 1995 explicitly teaches that *afp* encoded RNA is not detectable in samples containing all nucleated cells of blood, in accordance with the recitation: "*afp* RNA was not demonstrated in nuclear cell component of peripheral blood of healthy volunteers both by RT-PCR and even by nested PCR (Fig. 1)" (p. 815, first paragraph of "RESULTS" section and Fig. 1). As stated at p. 816 of Matsumura et al., 1995, the sensitivity of the assay employed therein corresponds to detection of 1-10 HepG2 *afp*-expressing control cells per 5 ml of blood (i.e. conservatively 10 cells per 5 ml of blood). Since there are 4,500 to 10,500 leukocytes per microliter of blood and conservatively 59% of those leukocytes are PMNCs, (refer to "Complete Blood Count" Table. Blood Disorders/Diagnosis, of Blood Disorders, In: The Merck Manual of Diagnosis and Therapy. Beers MH, et al. (eds), of record), i.e. conservatively 2.65 million PMNCs per ml of blood[9], this conservatively corresponds to a sensitivity of one HepG2 cell per 1.88 million PMNCs[10].

In contrast, the maximum sensitivity for detection of *afp* encoded RNA taught by Wong et al. is stated to be 1HepG2 cell per 1million PMNCs[11]. In fact, this sensitivity is actually the

[8] See p. 12 last paragraph and figure 3 which notes a sensitivity with as little as 0.156ul

[9] Using most conservative estimate of Merck Manual, 59% of leukocytes are PMNCs' therefore 45000000 million leukocytes is the equivalent of 45000000*.59 =2,655,000 PMNCs

[10]. [(10 HepG2 cells detected) / (5 ml blood)] × [(1 ml blood) / (2.65 million PMNCs)] =
1 HepG2 cell detected per 1.88 million PMNCs

[11] Assuming most conservative estimate as per Merck Manual 2006 of PMNCs (monocytes and lymphocytes) comprising 59% of

sensitivity of the assay for the detection of *alb* and is overstated for the ability to detect *afp* RNA which is demonstrated by Wong et al. to be 100 fold less sensitive than detection of *alb* (see Figure 1 and Figure 2 of Wong et al.). Nevertheless, the Matsumura et al. 1995 assay is still **at a minimum of 1.88 fold more sensitive** than the stated sensitivity of the assay taught by Wong et al. and yet fails to achieve detection of *afp* encoded RNA in healthy individuals despite having used methods which avoid the step of isolating a fraction of the white blood cells.

Matsumura et al., 1994 uses the assay developed in Matsumura et al., 1995 and using the same primers as taught in Matsumura et al., 1995 teaches analysis of *afp* and *alb* encoded RNA in the same type of samples (ie. nucleated blood cells as compared to fractionation used by Wong et al. to obtain PMNCs) (p. 1419, under "PATIENTS AND METHODS")). Matsumura et al., 1994 demonstrates that *afp* encoded RNA is not detectable in all 26 healthy subjects tested in accordance with the recitation: "alpha-fetoprotein mRNA was not demonstrated in 26 cases of normal healthy volunteers" (Abstract; and 2nd paragraph of "RESULTS" section and Table 2). Given the use of the same assay as Matsumura et al., 1995, it is fair to assume that the sensitivity of the Matsumura et al., 1994 and Matsumura et al., 1995 assays are equivalent and therefore Matsumura et al., 1994 is also more sensitive than Wong et al.

The greater sensitivity of the Mastumura et al. 1994 assay as compared with Wong et al. is further confirmed by the fact that Matsumura et al., 1994 has a two-fold higher frequency of detecting *afp* encoded RNA in blood of subjects who have HCC as compared with Wong et al. Thus, Matsumura et al., 1994 detects 17 of the 33 patients with HCC (52%; see Abstract and 2nd paragraph of "RESULTS" section), in comparison with the frequency achieved by the method of Wong et al. of detection in only 13 out of 64 HCC patients (20%; p. 631, column 2). As noted by Wong et al, this indicates that the method of Matsumura et al., 1994 has a significantly higher detection sensitivity than that taught by Wong et al. (ie. "the frequency of *alb* mRNA detection depends on the sensitivity of the assay used..." (Wong et al., p. 631, first paragraph of "Discussion" section)).

In addition, Matsumura et al., 1994 teaches that *alb* RNA <u>is</u> detected in unfractionated blood cells of the same non-disease control subjects even though *afp* RNA <u>is not</u> detectable (p. 1420, 1st paragraph of "RESULTS" section and Fig. 1). Such parallel detection of *alb* RNA in

leukocytes – detection in 1,000,000 PBMCs would be the equivalent of detection in 1,000,000 + (1,000,000*.41) = 1,410,000 leukocytes assuming remaining cells had no inhibitory effects.

whole leukocytes of non-disease subjects further serves as an internal positive control further supporting the reliability of the teaching of Matsumura et al., 1994 that *afp* encoded RNA is not detectable in samples which avoid isolating a fraction of the white blood cells.

Thus, Matsumura et al., 1994, **using techniques significantly more sensitive than the method utilized by Wong et al.** fails to achieve detection of *afp* RNA in RNA isolated from blood samples of healthy individuals using techniques comparable to Kephart.

Funaki et al. 1995 teaches use of a technique similar to the use of RNAgents® as described in the first method utilized by Kephart et al. Both Funaki and RNAgents®, use a mixture of guanidinium isothiocyanate, sodium citrate, sarcosyl (which is equivalent to n-lauryl sarcosine) and Beta-mercaptoethanol (see RNAgents technical bulletin, enclosed, and Funaki p. 1622, last paragraph)) to isolate RNA from a whole blood sample. Funaki et al. explicitly teaches that *afp* encoded RNA is not detectable in RNA isolated using these methods (p. 1627, last sentence), therefore Funaki et al. also demonstrates that methods which avoid fractionation of white blood cells do not result in the detection of *afp* encoded RNA .

Funaki et al. utilizes a method for detection far more sensitive than the methods utilized by Wong et al., therefore the reason for failure to detect *afp* RNA in healthy individuals in Funaki et al., cannot be because their assay is somehow not as affective as the assay utilized by Wong et al. In fact, Funaki et al. utilizes a highly sensitive 3-step (double-nested) PCR method (Abstract). As stated at p. 1625, last paragraph of Funaki et al., the sensitivity of the assay employed therein corresponds to detection of one HuH7 *afp* expressing control cell per milliliter of blood. This can be shown to conservatively correspond to a maximum detection sensitivity of **one HepG2 cell per 3.36 million PMNCs**, as follows:

(i) there are 4,500 to 10,500 leukocytes per microliter of blood (refer to "Complete Blood Count" Table. Blood Disorders/Diagnosis, of Blood Disorders, In: The Merck Manual of Diagnosis and Therapy. Beers MH, et al. (eds), of record), ie. conservatively 4.5 million leukocytes per ml of blood;

(ii) there are conservatively 2655 (59%) polymorphonuclear lymphocytes (PMNCs) per microliter of blood (refer to "Complete Blood Count" Table. Blood Disorders/Diagnosis, of Blood Disorders, In: The Merck Manual of Diagnosis and Therapy. Beers MH, et al. (eds), of record), ie. 2.655 million PMNCs per ml of blood; and

(ii) the prior art teaches in a side by side comparison that the number of *afp* RNA

transcripts per HepG2 cell is 7.6×104 and that the number of *afp* RNA transcripts per HuH7 cell is 6.0×104 (refer to p. 1387, Table 2 of Niwa Y. et al., 1996. Hepatology 23:1384). As such, detection of Afp RNA in HuH7 cells represents a more stringent assay than detection of HepG2 cells.

The minimum sensitivity achieved by Funaki et al. can, therefore, be conservatively calculated as 1 HepG2 cell per 3.36 million PMNCs which is more sensitive that the method used by Wong et al. (ie. 1 HepG2 cell per 1 million PMNCs)[12].

Thus, Funaki et al. fails to achieve detection of *afp* RNA in unfractionated blood cells of non-disease (non-HCC) subjects, despite using a method which is conservatively at least 3 fold more sensitive than the method employed by Wong et al., and which enables a 3.5-fold higher frequency of detection of *afp* RNA in blood of patients having HCC than is achieved by Wong et al.

Therefore, yet another method which avoids isolating PMNCs and is as sensitive as the method utilized by Wong et al., fails to enable detection of *afp* RNA in a healthy individual.

Lemoine et al. 1997 teaches isolation of whole nucleated blood cells using the methods of Macfarlane & Dahle (Nature 362:186) which isolates RNA directly from whole blood using a guanidinium isothyocianate or hot formamide mixture. Lemoine analyzes the ability to detect *afp* RNA in samples from 28 normal subjects (p. 44, col. 2, end of 1st parag.) via nested RT-PCR. Lemoine teaches that *afp* RNA is undetectable in whole leukocytes of all 28 normal control subjects tested (p. 48, col. 2, 1st paragraph). The frequency of detection of *afp* RNA in blood of patients having HCC achieved by the method of Lemoine et al. is 17% (Abstract), is also similar to the 20% frequency (13/64; p. 631, column 2) achieved by the method of Wong et al. suggesting the methods of Lemoine et al. and Wong et al. are of comparable sensitivity (see Wong et al. p. 631, first paragraph of "Discussion" section).

In addition, Lemoine et al. teaches that *alb* RNA is detected in the whole nucleated blood cells of the same normal control subjects in which *afp* RNA is not detectable (p. 48, col. 2, 1st paragraph). Such parallel detection of *alb* RNA in samples of whole blood from normal control subjects serves as a positive control further supporting the reliability of Lemoine et al.'s teaching

[12] [(1 HuH7 cell detected) / (1 ml blood)] × [(1 ml blood) / (2.655 million PMNCs)] × [(7.6×104 *a* mRNA transcripts) / (1 HepG2 cell)] / [(6.0×104 Afp mRNA transcripts) / (1 HuH7 cell)] = 1 HepG2 cell detected per 3.36 million PMNCs

that *afp* RNA is not detectable when utilizing RNA from whole blood samples as compared with isolating a fraction of white blood cells as taught by Wong et al.

Liu et al. 1998 teaches that *afp* RNA was not detectable via RT-PCR (Abstract) in the "nuclear cell component" of 10 healthy control subjects which were tested, in accordance with the recitation: "there were no clinical control patients whose samples showed detectable Afp mRNA" (Abstract).

The assay employed by Liu et al. is clearly more sensitive for detection of *afp* RNA than that taught by Wong et al. since Liu achieves detection of *afp* RNA in unfractionated blood cells of 53.8% of 50 HCC patients tested, whereas Wong et al. only achieves a 2.5-fold lower frequency of detection in 13/64 (20%) of HCC patients tested (p. 631, column 2). This conclusion is in accordance with the teaching of Wong et al., exemplified with respect to *alb*, wherein "the frequency of *alb* mRNA detection depends on the sensitivity of the assay used..." (Wong et al., p. 631, first paragraph of "Discussion" section).

Thus, Liu et al. fails to achieve detection of *afp* RNA in healthy individuals where the RNA has been isolated from whole blood so as to include all nucleated cells as compared with methods which isolate RNA from fractions of white blood cells as taught by Wong et al., despite using a significantly more sensitive method and enables a greater than 2.5-fold higher detection frequency of *afp* RNA in blood of HCC patients, than the method taught by Wong et al.

Thus, the prior art, exemplified in the extensive list of prior art references discussed above, directly, clearly and consistently teaches that *afp* encoded RNA is not detectable in RNA from whole blood samples of healthy subjects as is required by the combination of Kephart and Wong et al. so as to fall within the scope of the Applicant's claims. It is well understood that evidence in the prior art demonstrating failure of the combination is evidence to demonstrate that there would be no reasonable expectation of success (In re Rinehart, 531 F.2d 1048, 189 USPQ 143 (CCPA 1976)).

C. The combination of prior art teachings does not enable the claimed invention

(i) afp RNA levels in healthy individuals as taught by Wong et al. are not detectable in whole blood of healthy subjects using the methods of Kephart.

It can be clearly demonstrated that detection of *afp* RNA in PMNCs of healthy subjects as taught by Wong et al., is not enabled using the methods of Kephart. Namely, it can be shown that the Kephart methods provide limited sensitivity of even abundant transcripts like beta-actin, and, therefore, the Kephart methods would not be able to detect *afp* RNA in healthy individuals.

Lupberger et al. teaches that normal human leukocytes contain 491+/-97 beta-actin transcripts per single leukocyte, and hence teaches that such cells contain a conservative minimum of 394 transcripts per cell (see abstract). Assuming there are 4,500 leukocytes/ul of whole blood[13], this would indicate **1,770,000 beta-actin transcripts/ul of whole blood**[14].

Wong et al., teaches that maximal levels of *afp* RNA in healthy individuals corresponds to an unspecified value which is less than the equivalent of 10pg of HepG2 total RNA per 20ml of peripheral blood (p. 631); this value is herein taken to correspond to the ultra-conservative maximum of 10pg of HepG2 total RNA per 20ml of peripheral blood. Wong et al. further teaches that a single HepG2 cell contains 1.65pg of total RNA (p.629 Figure 1); therefore, there are the equivalent of 6.06 HepG2 cells in 20ml of peripheral blood in healthy individuals[15]. Matsumura *et al.*, 1995 (cited by Wong et al.) teaches that one HepG2 cell contains 20,000 *afp* RNA transcripts. Therefore, Wong et al. teaches that detection of *afp* RNA in healthy individuals is the equivalent of **6.06 transcripts /µl of whole blood**[16].

Kephart's most sensitive method (freeze-thaw), requires at least 0.156 microliters of blood to achieve detection of beta-actin transcripts (p.12), therefore Kephart's most sensitive method is only able to detect, at minimum, 276,120 transcripts.[17] Since there are only 6.06 *afp* transcripts/ul of whole blood, one would need to use approximately **46 milliliters** of whole blood to detect *afp* transcripts according to the methods of Kephart[18].

With respect to the other two methods taught by Kephart, each require at least 0.625 microliters of blood (i.e. a 4-fold greater volume) to achieve detection of target transcripts; the

[13] 4,500 to 10,500 leukocytes per microliter of blood, and hence a conservative minimum of 4.5 million leukocytes per milliliter of blood (refer to "Complete Blood Count" Table. Blood Disorders/Diagnosis, of Blood Disorders, In: The Merck Manual of Diagnosis and Therapy. Beers MH, et al. (eds), of record).

[14] [(394 beta-actin transcripts) / (1 leukocyte)] × [(4,500,000 leukocytes) / (1 ml healthy subject blood)] × [(1 ml) / (1000 microliters)] = 1,770,000 trancripts per microliter of blood from healthy subjects

[15] 10pg HepG2 total RNA/20ml blood / 1.65pg total RNA/1 HepG2 cell = 6.06HepG2cells/20ml blood

[16] [(6.06 HepG2 cells)/20ml blood)] × [(20,000 Afp transcripts) / (1 HepG2 cell)] × [(1 ml) / (1000 microliters)] = 6.06 Afp transcripts per microliter of blood from healthy subjects.

[17] 0.156ul whole blood x 1,770,000 transcripts beta-actin/ul whole blood = 276,120 transcripts

[18] (292,079) × (0.156 microliters minimum detection volume) × (1 ml / 1,000 microliters) = 46 ml blood.

volume of blood from healthy subjects which would be required to achieve detection of *afp* transcripts according to the teachings of Wong et al. would be **182 milliliters** of whole blood[19].

The above volumes are based on the teachings of Wong et al. and hence relate to amount of *afp* transcripts detectable in PMNCs. However, with respect to detection of *afp* transcripts in whole blood of healthy subjects as required by the Applicant's claims, the prior art has clearly failed to achieve such detection using methods having significantly greater sensitivities as compared to the method taught by Wong et al. as described above. Thus, it reasonably follows that correspondingly larger blood volumes than 46 ml and 184 ml, would be required to achieve detection using the freeze-thaw and non-freeze thaw methods of Kephart, respectively.

Clearly, since even 46 ml of blood is far beyond the range which can be analyzed using any of the methods taught by Kephart, which are explicitly directed to analysis of small volumes[20], and which are, moreover, demonstrably useful for analysis of blood volumes no greater than 10 microliters[21], modifying the teachings of Wong et al. by application of the teachings of Kephart would fail to enable detection of *afp* transcripts in whole blood of healthy subjects as would be required for the claims to be rendered obvious by Wong et al. in view of Kephart [Kumar, 418 F.3d at 1368 ("[I]n order to render an invention unpatentable for obviousness, the prior art must enable a person of ordinary skill to make and use the invention.") (citing Beckman Instruments, Inc. v. LKB Produkter AB, 892 F.2d 1547, 1551 (Fed. Cir. 1989)).

(ii) Afp RNA expressed in healthy individuals is a rare mRNA and Kephart teaches away from use of rare mRNAs.

Kephart detects beta-actin RNA in whole blood, but teaches away from the detection of rare mRNAs noting "the presence of increasing amounts of cell derived inhibitory agents at higher cell concentrations and the potential for increased background due to genomic contamination may interfere with the detection of rare mRNAs".

As described above, it can be shown that Wong et al. demonstrates there are 6.06 *afp* RNA transcripts per microliter of blood from healthy subjects. As is also demonstrated above, there are 1,770,000 beta-actin trancripts per microliter of blood from healthy subjects. Therefore, there are *292,079* more copies of beta-actin RNA than *afp* RNA as expressed in healthy subjects.

[19] (292,079) × (0.625 microliters minimum detection volume) × (1 ml / 1,000 microliters) = 182 ml blood.
[20] See p.11 "RNA isolation protocols that use small amounts (less than 100ul)";
[21] See Figure 2 lane 1 and Figure 3 lane 2.

Thus *afp* encoded RNA is a rare mRNA in comparison with beta-actin, and therefore it is improper to combine Kephart and Wong since Kephart teaches away from using rare mRNAs with his methods. ("It is improper to combine references where the references teach away from their combination"(In re Grasselli, 713 F.2d 731, 743, 218 USPQ 769, 779 (Fed. Cir. 1983)).

Double patenting rejections

While respectfully disagreeing with the contention that the claims can be rejected under double patenting rejections, Applicant will consider filing a terminal disclaimer should it be necessary upon the indication of allowable claims.

Conclusion

Applicant submits that all claims are allowable as written and respectfully request early favorable action by the Examiner. If the Examiner believes that a telephone conversation with Applicant's attorney/agent would expedite prosecution of this application, the Examiner is cordially invited to call the undersigned attorney/agent of record.

Respectfully submitted,

Date: June 8, 2007

Amy DeCloux 54849 for

Name: Kathleen Williams
Registration No.: 34, 380
Customer No.: 29933
Palmer & Dodge LLP
111 Huntington Avenue
Boston, MA 02199-7613
Tel: 617-239-0100

Encl.:

(a) Stedmen's Medical Dictionary 27th edition.

(b) Chest. 1998 Jul;114(1):89-93."In vivo detection of coronary artery anomalies in asymptomatic athletes by echocardiographic screening." Zeppilli P, dello Russo A, Santini C, Palmieri V, Natale L, Giordano A, Frustaci A.

(c) Byers T, Levin B, Rothenberger D, Dodd GD, Smith RA. American Cancer Society guidelines for screening and surveillance for early detection of colorectal polyps and cancer: update 1997. American Cancer Society Detection and Treatment Advisory Group on Colorectal Cancer CA Cancer J Clin. 1997;47:154-60

(d) Diabetes Care January 1999 Vol. 22 p. S20

(e) Canadian Medical Association Journal (1982) 127(7) "Cervical cancer screening programs, summary of the 1982 Canadian task force report

(f) Du et al. (2006) Genomic profiles for human peripheral blood T cells, B cells, natural killer cells, monocytes, and polymorphonuclear cells: Comparisons to ischemic stroke, migraine and Tourette syndrome) Genomics 87 693-703)

(g) Funaki et al. 1995. Highly-sensitive identification of alpha-fetoprotein mRNA in circulating peripheral blood of hepatocellular carcinoma patients. Life Sci. 57:1621;

(h) Lemoine et al. 1997. Prospective evaluation of circulating hepatocytes by alpha-fetoprotein mRNA in humans during liver surgery. Ann Surg. 226:43;

(i) Matsumura et al. 1994. Detection of alpha-fetoprotein mRNA, an indicator of hematogenous spreading hepatocellular carcinoma, in the circulation: a possible predictor of metastatic hepatocellular carcinoma. Hepatology. 20:1418;

(j) Matsumura et al. 1995. Sensitive assay for detection of hepatocellular carcinoma associated gene transcription (alpha-fetoprotein mRNA) in blood. Biochem Biophys Res Commun. 207:813;

(k) Macfarlane DE, Dahle CE., 1993. Nature. 362:186-8. Isolating RNA from whole blood--the dawn of RNA-based diagnosis?

(l) Chomczynski P, and Sacchi N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 162(1):156-9.

(m) Lupberger J, Kreuzer KA, Baskaynak G, Peters UR, le Coutre P, Schmidt CA.Quantitative analysis of beta-actin, beta-2-microglobulin and porphobilinogen deaminase mRNA and their comparison as control transcripts for RT-PCRMol Cell Probes. 2002 Feb;16(1):25-30

(n) Promega Technical Bulletin, RNAgents® Total RNA Isolation System (March 2005) Part #TB087. Pages 1-20

Abstracts Referred To

Presse Med. 1983 Jan 22;12(3):147-51. "Asymptomatic thyroid dysfunction. Routine detection using the free thyroxine" Schlienger JL, Heist D, Demangeat C, Sapin R, Stephan F.

Free plasma thyroxine index (FT4I) and plasma concentrations of triiodothyronine (T3) were routinely determined on admission in 1862 hospital patients without signs or history of thyroid dysfunction. Total thyroxine (T4) and FT4I values were beyond confidence limits in 16.3% and 7.5% respectively of the patients, whereas T3 values were low in 23%. Among the 84 patients with high FT4I, 31 had clinically unsuspected hyperthyroidism confirmed by the TRH test (T3 increased in 66% of the cases). Among the 46 patients with low FT4I, 14 had demonstrable hypothyroidism (low T3 in 65% of the cases). In this population, the prevalence of proven thyroid dysfunction without clinical symptoms was 1.66% for hyperthyroidism and 0.75% for hypothyroidism. The diagnostic value of normal FT4I was estimated at 50%. The persistence of an abnormal FT4I on a second determination indicated the presence of hyperthyroidism in 72% of patients with high FT4I values and of hypothyroidism in 74% of patients with low FT4I values. In doubtful cases, TSH assays or TRH tests led to the concept of "transient" dysthyroidism, and the potential total prevalence of routinely discovered dysthyroidism could be estimated at 3.54% including 2.47% for hyperthyroidism. The latter occurred in 95% of people older than 50, with a sex ratio of 0.94. The cost of diagnosis for each new case clinically unsuspected hyperthyroidism is 1200 Z, but this could be reduced to 450 Z if only patients over 50 years of age were investigated and if FT4I determinations were replaced by free T4 determinations.

PMID: 6220353 [PubMed - indexed for MEDLINE]

Liu Y et al., 1998. [The detection of circulating hepatocellular carcinoma cells in peripheral venous blood by reverse transcription-polymerase chain reaction and its clinical significance]. Zhonghua Wai Ke Za Zhi. 36:608-10. [Article in Chinese]

OBJECTIVE: To detect circulating hepatocellular carcinoma by demonstrating hepatocellular carcinoma cells-associated mRNA in the nuclear cell component of peripheral blood (PBL). METHOD: Peripheral blood (5 ml) samples were obtained from 93 patients with hepatocellular carcinoma (HCC) and from 37 controls (15 controls with liver cirrhosis after hepatitis B, 12 chronic hepatitis B, and 10 normal liver function). To identify HCC cells in peripheral blood, liver-specific human alpha-fetoprotein (AFP) mRNA was amplified from total RNA extracted from whole blood by reverse transcription-polymerase chain reaction. RESULT: AFP mRNA was detected in 50 blood samples from the patients with HCC (53.8%). In contrast, there were no clinical control patients whose samples showed detectable AFP mRNA in PBL. The presence of AFP mRNA in blood seemed to be correlated with the stage (by TNM classification) of HCC, the serum AFP value, and the presence of intrahepatic metastasis, portal vein thrombosis, tumor diameter and/or distant metastasis. AFP mRNA was detected in the blood of 21 patients showing metastasis at extrahepatic organs (100%) in contrast to 29 of 72 patients without metastasis (40.3%). CONCLUSION: The presence of AFP mRNA in peripheral blood may be an indicator of malignant hepatocytes, which might predict hematogenous spreading metastasis of tumor cells in patients with HCC.

PMID: 11825477 [PubMed - indexed for MEDLINE]

Arch Surg. 1986 Nov;121(11):1347-52.

Colorectal cancer. A blueprint for disease control through screening by primary care physicians.

Wanebo HJ, Fang WL, Mills AS, Zfass AM.

The Virginia Colorectal Cancer Control Project is a statewide effort to reduce morbidity and mortality from colorectal cancer by stimulating the adoption of screening and early detection practices by primary care physicians. The project emphasizes use of the three-day fecal occult blood test, digital rectal examination, and endoscopy. Recruitment strategies included personal contact, newsletters, journal articles, and screening workshops. Of the 33 318 patients screened over 26 months, positive fecal occult blood test reactions were recorded in 3.3% of asymptomatic patients and in 14.8% of symptomatic patients. Polyps were found in 149 and cancer was diagnosed in 94 patients of whom one third were asymptomatic. Eighty percent of the latter had Dukes' A and B lesions, 12% had Dukes' C lesions, and 8% had Dukes' D lesions. In contrast, only 36% of the symptomatic cancers were Dukes' A and B lesions, and 69% were Dukes' C and D lesions. These results suggest that primary care physicians can be effective in the screening and detection of precancerous polyps and early-staged colorectal cancers.

STEDMAN'S Medical Dictionary

27th Edition

Illustrated in Color

LIPPINCOTT WILLIAMS & WILKINS
A Wolters Kluwer Company
Philadelphia • Baltimore • New York • London
Buenos Aires • Hong Kong • Sydney • Tokyo

A

Senior Managing Editor: Maureen Barlow Pugh
Managing Editor: Barbara Werner
New Terms Editor: Thomas W. Filardo, MD
Copy Editors: Peter W. Binns, Linda G. Francis, Raymond Lukens, Bonnie Montgomery
Chief On-Line Editor: Barbara L. Ferretti
On-Line Editors: Kathryn J. Cadle, Dana Workman
Proofreaders: Peter W. Binns; David A. Bloom, MD; Alfred J. Bollet, MD; Ted Burk; Regina Lavette Davis; John A. Day, Jr., MD, FCCP; Richard Diamanti; John H. Dirckx, MD; Thomas W. Filardo, MD; Linda G. Francis; John M. Last, MD, FRACP, FRCPC; Raymond Lukens; Kate Mason, CMT; Joan Sarchese
Database Programmers: Dave Marcus, Lexi-Comp Inc., Hudson, OH
Art Director: Jonathan Dimes
Illustrations: Neil O. Hardy
Additional artwork by: Mary Anna Barratt-Dimes, Kathryn Born, Rob Duckwall, Timothy Hengst, Mikki Senkarik, Michael Schenk, Larry Ward
Graphic preparation assistance: Susan Caldwell, Jennifer Clements, Thomas Dolan, Christina Nihira
Design: Dan Pfisterer

Database design by Lexi-Comp Inc., Hudson, OH
Printed in the United States of America by World Color, Inc.

Library of Congress Cataloging-in-Publication Data

Stedman, Thomas Lathrop, 1853-1938.
Stedman's medical dictionary.—27th ed.
p.; cm.
ISBN 0-683-40007-X (regular)—ISBN 0-683-40008-8 (deluxe)
1. Medicine—Dictionaries. I. Title: Medical dictionary. II. Title.
[DNLM: 1. Medicine—Dictionary—English. W 13 S812m 1999]
R121 .S8 1999
610'.3—dc21

99-056094

00 01 02 03 04 05
1 2 3 4 5

Indian s., SYN epidemic gangrenous *proctitis*.
Jamaican vomiting s., SYN ackee *poisoning*.
milk s., a disease of humans caused by ingesting contaminated milk from cows suffering from trembles; clinical manifestations include severe vomiting, labored breathing, delirium, convulsions, coma, and death; recovery from nonlethal illness is slow. SYN lactimorbus.
morning s., the nausea and vomiting of early pregnancy. SYN morning vomiting, nausea gravidarum.
motion s., the syndrome of pallor, nausea, weakness, and malaise, which may progress to vomiting and incapacitation, caused by stimulation of the semicircular canals during travel or motion as on a boat, plane, train, car, swing, or rotating amusement ride. SYN kinesia.
mountain s., SYN altitude s.
radiation s., a systemic condition caused by substantial whole-body irradiation, seen after nuclear explosions or accidents, rarely after radiotherapy. Manifestations depend on dose, ranging from anorexia, nausea, vomiting, and mild leukopenia, to thrombocytopenia with hemorrhage, severe leukopenia with infection, anemia, central nervous system damage, and death. SYN radiation poisoning.
sea s., motion s. occurring in boat travelers.
serum s., an immune complex disease appearing some days (usually 1–2 weeks) after injection of a foreign serum or serum protein, with local and systemic reactions such as urticaria, fever, general lymphadenopathy, edema, arthritis, and occasionally albuminuria or severe nephritis; originally described in patients receiving serotherapy. The term is sometimes used for clinically similar allergic reactions to drugs. SYN serum disease, serum reaction.
sleeping s., SEE Gambian *trypanosomiasis*, Rhodesian *trypanosomiasis*.
space s., dizziness as result of changes in inner ear resulting from absence of gravity. SYN physiologic vertigo.
West African sleeping s., SYN Gambian *trypanosomiasis*.

side (sīd). One of the two lateral margins or surfaces of a body, midway between the front and back. [A.S. *side*]
balancing s., in dentistry, the nonfunctioning s. from which the mandible moves during the working bite.
working s., in dentistry, the lateral segment of a dentition toward which the mandible is moved during occlusal function.

side ef·fect. A result of drug or other therapy in addition to or in extension of the desired therapeutic effect; usually but not necessarily, connoting an undesirable effect. Although technically the therapeutic effect carried beyond the desired limit (e.g., a hemorrhage from an anticoagulant) is a s. e., the term more often refers to pharmacologic results of therapy unrelated to the usual objective (e.g., a development of signs of Cushing syndrome with steroid therapy).

sid·er·a·tion (sid-er-ā'shŭn). Any sudden attack, as of apoplexy. [L. *sideror*, pp. *sideratus*, to be blasted or palsied by a constellation, fr. *sidus* (*sider-*), a constellation, the heavens]

△**sidero-.** Iron. [G. *sidēros*]

sid·er·o·blast (sid'er-ō-blast). An erythroblast containing granules of ferritin stained by the Prussian blue reaction. [sidero- + G. *blastos*, germ]

sid·er·o·cyte (sid'er-ō-sīt). An erythrocyte containing granules of free iron, as detected by the Prussian blue reaction, in the blood of normal fetuses, where they constitute from 0.10–4.5% of the erythrocytes. [sidero- + G. *kytos*, cell]

sid·er·o·fi·bro·sis (sid'er-ō-fī-brō'sis). Fibrosis associated with small foci in which iron is deposited.

sid·er·og·en·ous (sid-er-oj'ĕ-nŭs). Iron forming. [sidero- + G. *-gen*, producing]

sid·er·o·pe·nia (sid'er-ō-pē'nē-ă). An abnormally low level of serum iron. [sidero- + G. *penia*, poverty]

sid·er·o·pe·nic (sid'er-ō-pē'nik). Characterized by sideropenia.

sid·er·o·phage (sid'er-ō-fāj). SYN siderophore. [sidero- + G. *phagō*, to eat]

sid·er·o·phil, sid·er·o·phile (sid'er-ō-fil, -fīl). 1. Absorbing iron. SYN siderophilous. 2. A cell or tissue that contains iron. [sidero- + G. *philos*, fond]

sid·er·oph·i·lins (sid-er-ō-fil'in, -of'i-lin). Nonheme, iron-binding proteins; there are three central classes of s.: transferrin (1) in vertebrate blood), lactoferrin (in mammalian milk and other secretions), and conalbumin or ovotransferrin (avian blood and avian egg white).

sid·er·oph·i·lous (sid-er-of'i-lŭs). SYN siderophil (1).

sid·er·o·phore (sid'er-ō-fōr). A large extravasated mononuclear phagocyte containing granules of hemosiderin, found in the sputum or in the lungs of individuals with longstanding pulmonary congestion from left ventricular failure. SEE ALSO heart failure cell. SYN siderophage. [sidero- + G. *phoros*, bearing]

sid·er·o·sil·i·co·sis (sid'er-ō-sil'i-kō'sis). Silicosis due to inhalation of dust containing iron and silica. SYN silicosiderosis. [sidero- + silicosis]

sid·er·o·sis (sid-er-ō'sis). 1. A form of pneumoconiosis due to the presence of iron dust. 2. Discoloration of any part by deposition of a pigment containing iron; usually called hemosiderosis. 3. An excess of iron in the circulating blood. 4. Degeneration of the retina, lens, and uvea as a result of the deposition of intraocular iron. [sidero- + G. *-osis*, condition]
pulmonary s., SYN *pneumoconiosis* siderotica.

sid·er·ot·ic (sid-er-ot'ik). Related to siderosis; pigmented by iron or containing an excess of iron.

SIDS Acronym for sudden infant death *syndrome*.

Siegert, Ferdinand, German pediatrician, 1865–1946. SEE S. sign.

Siegle, Emil, German otologist, 1833–1900. SEE S. *otoscope*.

sie·mens (S) (sē'menz). The SI unit of electrical conductance; the conductance of a body with an electrical resistance of 1 ohm, allowing 1 ampere of current to flow per volt applied; equal to 1 mho. SYN mho. [Sir William *Siemens*, Ger. born British engineer, 1823–1883]

Siemerling, Ernst, German physician, 1857–1931.

sieve (siv). A meshed or perforated device for separating fine particles from coarser ones. [O.E. *sive*]
molecular s., a gel-like material with pore sizes of such ranges as to exclude molecules above certain sizes; used in fractionating or purifying macromolecules.

sie·vert (Sv) (sē'vert). The SI unit of ionizing radiation effective dose, equal to the absorbed dose in gray, weighted for both the quality of radiation in question and the tissue response to that radiation. The unit is the joule per kilogram and 1 Sv = 100 rem. SEE effective *dose*, equivalent *dose*.

SIF Abbreviation for somatotropin release-inhibiting *factor*.

Sig. Abbreviation for L. *signa*, label, write, or *signetur*, let it be labeled.

Siggaard-Andersen, Ole, Danish clinical biochemist, *1932. SEE Siggaard-Andersen *nomogram*.

sigh (sī). 1. An audible inspiration and expiration under the influence of some emotion. 2. To perform such an act. [A.S. *sican*]

sight (sīt). The ability or faculty of seeing. SEE ALSO vision. [A.S. *gesihth*]
day s., SYN nyctalopia.
far s., SYN hyperopia.
long s., SYN hyperopia.
near s., SYN myopia.
night s., SYN hemeralopia.
second s., improved near vision in the aged as a result of increased refractivity of the nucleus of the lens causing myopia. SYN senile lenticular myopia.
short s., SYN myopia.

sig·ma (sig'mă). The 18th letter of the Greek alphabet, σ.

sig·ma·tism (sig'mă-tizm). SYN lisping. [G. *sigma*, the letter S]

sig·moid (sig'moyd). Resembling in outline the letter S or one of the forms of the Greek sigma. [G. *sigma*, the letter S, + *eidos*, resemblance]

△**sigmoid-.** SEE sigmoido-.

sig·moi·dec·to·my (sig-moy-dek'tō-mē). Excision of the sigmoid colon. [sigmoid- + G. *ektomē*, excision]

sig·moid·ic·i·ty (sig'moyd-i-sa-tē). Describing an S-shaped curve; e.g., shape of enzyme-kinetic curves for enzymes displaying positive homotropic cooperativity.

sig·moid·i·tis (sig-moy-dī'tis). Inflammation of the sigmoid colon. [sigmoid- + G. *-itis*, inflammation]

△**sigmoido-, sigmoid-.** Sigmoid, usually the sigmoid colon. [G. *sigma*, the letter σ, + *eidos*, resemblance]

sig·moi·do·pexy (sig-moy'dō-pek-sē). Operative attachment of the sigmoid colon to a firm structure to correct rectal prolapse. [sigmoido- + G. *pēxis*, fixation]

sig·moi·do·proc·tos·to·my (sig-moy'dō-prok-tos'tō-mē). Anastomosis between the sigmoid colon and the rectum. SYN sigmoidorectostomy. [sigmoido- + G. *prōktos*, anus, + *stoma*, mouth]

sig·moi·do·rec·tos·to·my (sig-moy'dō-rek-tos'tō-mē). SYN sigmoidoproctostomy.

sig·moi·do·scope (sig-moy'dō-skōp). An endoscope for viewing the lumen of the sigmoid colon. SYN sigmoscope. [sigmoido- + G. *skopeō*, to view]

sig·moi·dos·co·py (sig'moy-dos'kŏ-pē). Inspection, through an endoscope, of the interior of the sigmoid colon.

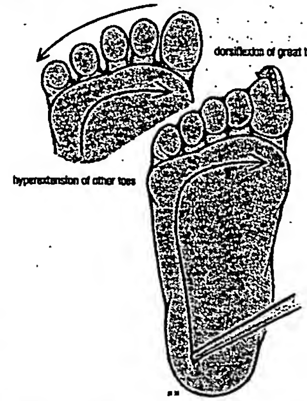
sig·moi·dos·to·my (sig'moy-dos'tō-mē). Establishment of an artificial anus by opening into the sigmoid colon. [sigmoido- + G. *stoma*, mouth]

sig·moi·dot·o·my (sig'moy-dot'ō-mē). Surgical opening of the sigmoid. [sigmoido- + G. *tomē*, incision]

sig·mo·scope (sig'mō-skōp). SYN sigmoidoscope.

SIGN

sign (sīn). 1. Any abnormality indicative of disease, discoverable on examination of the patient; an objective indication of disease, in contrast to a symptom, which is a subjective indication of disease. 2. An abbreviation or symbol. 3. In psychology, any object or artifact (stimulus) that represents a specific thing or conveys a specific idea to the person who perceives it. [L. *signum*, mark]
Aaron s., in acute appendicitis, a referred pain or feeling of distress in the epigastrium or precordial region on continuous firm pressure over the McBurney point.
Abadie s. of tabes dorsalis, insensibility to pressure over the tendo achillis.
Abrahams s., an obsolete sign. (1) rales and other adventitious sounds, changes in the respiratory murmurs, and increase in the whispered sound can be heard on auscultation over the acromial end of the clavicle some time before they become audible at the apex; heard primarily in pulmonary tuberculosis affecting the apical portion of the lung; (2) a dull-flat note, i.e., one between the normal dullness at the right apex and absolute flatness, heard on percussion in that region, indicating progress from incipient to advanced tuberculosis.
accessory s., a finding frequently but not consistently present in a disease. SYN assident s.
antecedent s., SYN prodromic s.
assident s., SYN accessory s.
Auenbrugger s., an epigastric prominence seen in cases of marked pericardial effusion.
Aufrecht s., diminished or noisy breath sounds in the trachea just above the jugular notch, in cases of stenosis.
Auspitz s., a finding typical of psoriasis in which removal of a scale leads to pinpoint bleeding.
Babinski s., (1) extension of the great toe and abduction of the other toes instead of the normal flexion reflex to plantar stimulation, considered indicative of pyramidal tract involvement ("positive" Babinski); SYN Babinski phenomenon, Babinski reflex, great-toe reflex, paradoxical extensor reflex, toe phenomenon. (2) in hemiplegia, weakness of the platysma muscle on the affected side, as is evident in such actions as blowing or opening the mouth; (3) when the patient is lying supine, with arms crossed on the front of the chest, and attempts to assume the sitting posture, the thigh on the side of an *organic* paralysis is flexed and the heel raised, whereas the limb on the sound side remains flat; (4) in hemiplegia, the forearm on the affected side turns to a pronated position when placed in a position of supination.



Babinski sign: on stimulation of sole of foot, extension of the great toe with fanning of the other toes is a normal response up to six months of age, but abnormal thereafter; a positive response is considered indicative of pyramidal tract involvement

Baccelli s., an obsolete s.: good conduction of the whisper in nonpurulent pleural effusions. SYN aphonic pectoriloquy.
Ballance s., the presence of a dull percussion note in both flanks, constant on the left side but shifting with change of position on the right, said to indicate ruptured spleen; the dullness is due to the presence of fluid blood on the right side but coagulated blood on the left.
Bamberger s., (1) jugular pulse in tricuspid insufficiency; (2) SYN allochiria; (3) dullness on percussion at the angle of the scapula, clearing up as the patient leans forward, indicating pericarditis with effusion. SYN Bamberger-Pins-Ewart s.
Bamberger-Pins-Ewart s., SYN Bamberger s.
banana s., the abnormal curvature of the cerebellum noted on ultrasound imaging in a fetus with Arnold-Chiari *malformation*.
Bárány s., in cases of ear disease, in which the vestibule is healthy, injection into the external auditory canal of water below the body temperature will cause rotatory nystagmus toward the opposite side; when the injected fluid is above the body temperature the nystagmus will be toward the injected side; if the labyrinth is diseased or nonfunctional there may be diminished or absent nystagmus.
Barré s., a hemiplegic placed in the prone position with the limbs flexed at the knees is unable to maintain the flexed position on the side of the lesion but extends the leg.
Bassler s., in chronic appendicitis, pinching the appendix between the thumb and the iliacus muscle causes sharp pain.
Bastedo s., an obsolete s.: in chronic appendicitis, pain and tenderness in the right iliac fossa on inflation of the colon with air.
Battle s., postauricular ecchymosis in cases of fracture of the base of the skull.

sympathetectomy, sympathicectomy. [sympath- + G. *ektomē*, excision]

chemical s., destruction of the periarterial sympathetic nerves, as in Doppler operation, by a corrosive such as phenol.

periarterial s., sympathetic denervation by arterial decortication. SYN histonectomy, Leriche operation.

presacral s., SYN presacral *neurectomy*.

sym·pa·the·tec·to·my (sim-pă-thĕ-tek'tō-mē). SYN sympathectomy.

sym·pa·thet·ic (sim-pă-thet'ik). **1.** Relating to or exhibiting sympathy. **2.** Denoting the sympathetic part of the autonomic nervous system. SYN sympathic. [G. *sympathētikos*, fr. *sympatheō*, to feel with, sympathize, fr. *syn*, with, + *pathos*, suffering]

sym·pa·thet·o·blast (sim-pă-thet'ō-blast). SYN sympathoblast.

sym·pa·thic (sim-path'ik). SYN sympathetic.

sym·path·i·cec·to·my (sim-path'i-sek'tō-mē). SYN sympathectomy.

△sympathico-. SEE sympath-.

sym·path·i·co·blast (sim-path'i-kō-blast). SYN sympathoblast.

sym·path·i·co·neu·ri·tis (sim-path'i-kō-noo-rī'tis). Inflammation of the autonomic nerves.

sym·path·i·cop·a·thy (sim-path-i-kop'ă-thē). A disease resulting from a disorder of the autonomic nervous system. [sympathico- + G. *pathos*, suffering]

sym·path·i·co·to·nia (sim-path'i-kō-tō'nē-ă). A condition in which there is increased tonus of the sympathetic system and a marked tendency to vascular spasm and high blood pressure; opposed to vagotonia. [sympathico- + G. *tonos*, tone, tension]

sym·path·i·co·ton·ic (sim-path'i-kō-ton'ik). Relating to or characterized by sympathicotonia.

sym·path·i·co·trip·sy (sim-path'i-kō-trip'sē). Operation of crushing the sympathetic ganglion. [sympathico- + G. *tripsis*, a rubbing]

sym·pa·thin (sim'pă-thin). The substance diffusing into circulation from sympathetic nerve terminals when they are active. The term was introduced by W.B. Cannon, who thought that this substance differed from the mediator produced by the nerve ending (now known to be incorrect); the mediator itself (norepinephrine) diffuses into circulation. SYN sympathetic hormone.

sym·pa·thism (sim'pă-thizm). SYN suggestibility. [G. *sympatheia*, sympathy]

sym·pa·thiz·er (sim'pă-thī-zer). **1.** An eye affected with sympathetic ophthalmia. **2.** One who exhibits sympathy.

△sympatho-. SEE sympath-.

sym·pa·tho·ad·re·nal (sim'pă-thō-ă-drē'năl). Relating to the sympathetic part of the autonomic nervous system and the medulla of the adrenal gland, as the postganglionic neurons.

sym·pa·tho·blast (sim'pă-thō-blast). A primitive cell derived from the neural crest glia; with the pheochromoblasts, s.'s enter into the formation of the adrenal medulla and sympathetic ganglia. SYN sympathetoblast, sympathicoblast. [sympatho- + G. *blastos*, germ]

sym·pa·tho·go·nia (sim'pă-thō-gō'nē-ă). The completely undifferentiated cells of the sympathetic nervous system. [sympatho- + G. *gonē*, seed]

sym·pa·tho·lyt·ic (sim'pă-thō-lit'ik). Denoting antagonism to or inhibition of adrenergic nerve activity. SEE ALSO adrenergic blocking *agent*, antiadrenergic. [sympatho- + G. *lysis*, a loosening]

sym·pa·tho·mi·met·ic (sim'pă-thō-mi-met'ik). Denoting mimicking of action of the sympathetic system. SEE ALSO adrenomimetic. [sympatho- + G. *mimikos*, imitating]

sym·pa·thy (sim'pă-thē). **1.** The mutual relation, physiologic or pathologic, between two organs, systems, or parts of the body. **2.** Mental contagion, as seen in mass hysteria or in the yawning induced by seeing another person yawn. **3.** An expressed sensitive appreciation or emotional concern for and sharing of the mental and emotional state of another person. Cf. empathy (1). [G. *sympatheia*, fr. *syn-* + *pathos*, suffering]

sym·per·i·to·ne·al (sim'per-i-tō-nē'ăl). Relating to the surgical induction of adhesion between two portions of the peritoneum.

sym·pha·lan·gism, sym·pha·lan·gy (sim-fal'an-jizm, sim-fal'an-jē). **1.** SYN syndactyly. **2.** Ankylosis of the finger or toe joints. [sym- + phalanx]

sym·phys·i·al, sym·phys·e·al (sim-fiz'ē-ăl). Grown together; relating to a symphysis; fused. SYN symphysic.

sym·phys·ic (sim-fiz'ik). SYN symphysial.

sym·phys·i·on (sim-fiz'ē-on). A craniometric point, the most anterior point of the alveolar process of the mandible.

sym·phys·i·o·tome, sym·phys·e·o·tome (sim-fiz'ē-ō-tōm). Instrument for use in symphysiotomy.

sym·phys·i·ot·o·my, sym·phys·e·ot·o·my (sim-fiz-ē-ot'ō-mē). Division of the pubic joint to increase the capacity of a contracted pelvis sufficiently to permit passage of a living child. SYN synchondrotomy. [symphysis + G. *tomē*, incision]

sym·phy·sis, gen. **sym·phy·ses** (sim'fi-sis, -sēz) [TA]. **1** [NA]. Form of cartilaginous joint in which union between two bones is effected by means of fibrocartilage. SYN amphiarthrosis. **2.** A union, meeting point, or commissure of any two structures. **3.** A pathologic adhesion or growing together. SYN secondary cartilaginous joint [TA]. [G. a growing together]

intervertebral s. [TA], the union between adjacent vertebral bodies composed of the nucleus pulposus, annular ligament, and the anterior and posterior longitudinal ligaments. SYN s. intervertebralis [TA].

s. intervertebra'lis [TA], SYN intervertebral s.

s. mandib'ulae [TA], SYN mandibular s.

mandibular s. [TA], the fibrocartilaginous union of the two halves of the mandible in the fetus; it becomes an osseous union during the first year. SYN s. mandibulae [TA], mental s., s. mentalis, s. menti.

manubriosternal s. [TA], the later union, by fibrocartilage, of the manubrium and the body of the sternum; it begins as a synchondrosis and becomes a symphysis, occasionally fusing to become a synostosis. SYN s. manubriosternalis [TA], sternomanubrial junction.

s. manubriosterna'lis [TA], SYN manubriosternal s.

mental s., SYN mandibular s.

s. menta'lis, SYN mandibular s.

s. men'ti, SYN mandibular s.

pericardial s., adhesion between the parietal and visceral layers of the pericardium.

pubic s. [TA], the firm fibrocartilaginous joint between the two pubic bones. SYN s. pubica [TA], s. pubis.

s. pu'bica [TA], SYN pubic s.

s. pu'bis, SYN pubic s.

s. sacrococcyg'ea, SYN sacrococcygeal *joint*.

s. xiphosternalis [TA], SYN xiphisternal *joint*.

sym·plas·mat·ic (sim-plaz-mat'ik). Relating to the union of protoplasm as in giant cell formation. [G. *sym- plassō*, to mold together]

sym·plast (sim'plast). A multinucleated cell that has formed by fusion of separate cells. [sym- + G. *plastos*, formed]

sym·po·dia (sim-pō'dē-ă). Condition characterized by union of the feet. SEE ALSO sirenomelia, sympus. [sym- + G. *pous*, foot]

sym·port (sim'pōrt). Coupled transport of two different molecules or ions through a membrane in the same direction by a common carrier mechanism (symporter). Cf. antiport, uniport. [sym- + L. *porto*, to carry]

sym·port·er (sim-pōrt'er). The protein responsible for mediating symport.

symp·tom (simp'tŏm). Any morbid phenomenon or departure from the normal in structure, function, or sensation, experienced by the patient and indicative of disease. SEE ALSO phenomenon (1), reflex (1), sign (1), syndrome. [G. *symptōma*]

abstinence s.'s, SYN withdrawal s.'s.

accessory s., a s. that usually but not always accompanies a certain disease, as distinguished from a pathognomonic s. SYN assident s., concomitant s.

accidental s., any morbid phenomenon coincidentally occurring in the course of a disease, but having no relation with it.

B

CHEST

Official publication of the American College of Chest Physicians

CHEST ONLINE

In vivo detection of coronary artery anomalies in asymptomatic athletes by echocardiographic screening

P Zeppilli, A dello Russo, C Santini, V Palmieri, L Natale, A Giordano and A Frustaci

Chest 1998;114;89-93

AMERICAN COLLEGE OF CHEST PHYSICIANS®

In Vivo Detection of Coronary Artery Anomalies in Asymptomatic Athletes by Echocardiographic Screening*

Paolo Zeppilli, MD; Antonio dello Russo, MD; Cesare Santini, MD; Vincenzo Palmieri, MD; Luigi Natale, MD; Alessandro Giordano, MD; and Andrea Frustaci, MD, FCCP

Background: **Anomalous origin of coronary arteries (AOCA) is a rare congenital disease. Although it may have a benign course, it has been identified as a frequent cause of sports-related sudden death. Unfortunately, *in vivo* detection of AOCA is not easy, as individuals with this anomaly often are asymptomatic and show no signs of myocardial ischemia. Presently, transthoracic two-dimensional echocardiography (TTE) is the only noninvasive, widely available tool to visualize the ostia and first tracts of coronary arteries.**

Objective: **To assess the efficacy of TTE in the screening of AOCA in a large athletic population.**

Study design: **In a prospective study, we assessed the ostia and first tracts of coronary arteries in 3,650 subjects (mean age, 30±12 years) practicing different sports at various competitive levels. Subjects underwent a TTE examination in our laboratory for scientific or diagnostic purposes.**

Results: **Technically satisfactory echocardiograms were obtained in 3,504 subjects (96%); a clear visualization of the ostia and first tracts of both coronary arteries was obtained in 3,150 cases (90%). Three asymptomatic athletes (0.09%) were suspected to have an AOCA; two with a right coronary artery origin from the left sinus, and one with a left coronary artery origin from the right sinus. Diagnosis was confirmed by coronary angiography.**

Conclusions: **Our study indicated that AOCA is rare in asymptomatic athletes. Systematic and accurate exploration of coronary anatomy in athletes referred for a diagnostic TTE examination may be useful in identifying those with AOCA.** ***(CHEST 1998; 114:89-93)***

Key words: anomalous origin of coronary artery; athletes; echocardiography; sudden death

Abbreviations: AOCA=anomalous origin of coronary arteries; LCA=left coronary artery; RCA=right coronary artery; TTE=transthoracic two-dimensional echocardiography

Anomalous origin of coronary arteries (AOCA) is a rare congenital disease found on approximately 0.6 to 1% of all coronary angiograms and in 0.3% of all autopsies.[1-3] Although an AOCA may have a completely benign and asymptomatic course, it has been identified as a cause for sports-related and non-sports-related sudden death in young people and athletes.[4-6]

Unfortunately, *in vivo* detection of AOCA is not always easy, because subjects with this anomaly are often asymptomatic, and symptoms attributable to myocardial ischemia are observed only in about 30% of cases.[4] Presently, transthoracic two-dimensional echocardiography (TTE) offers a reliable, noninvasive means of visualizing the ostia and first tracts of both coronary arteries, especially in children and young adults.[7] Visualization of these structures by TTE is easier in athletes because of their favorable chest conformation, the prolonged diastolic time due to bradycardia, and a real increase in coronary artery size due to training.[8-10] The real prevalence of AOCA in athletes is not known but it is probably very low; Pelliccia et al[9] found no cases of AOCA in a large group of asymptomatic elite athletes prospectively studied by TTE.

Since 1986, in our Sports Medicine Laboratory, routine TTE examination of all subjects practicing sports has included a systematic search of the ostia and the first tracts of both coronary arteries. Using this approach, we were able to identify three athletes

*From the Centro Studi di Medicina dello Sport, Istituto di Medicina Interna e Geriatria (Drs. Zeppilli, dello Russo, Santini, and Palmieri), the Istituto di Radiologia (Dr. Natale), the Istituto di Medicina Nucleare (Dr. Giordano), and the Istituto di Cardiologia (Dr. Frustaci), Università Cattolica del Sacro Cuore, Roma, Italy.

Manuscript received September 26, 1997; revision accepted January 22, 1998.

Reprint requests: Dott. Andrea Frustaci, Istituto di Cardiologia, Università Cattolica del Sacro Cuore, Largo Gemelli 8, 00168 Rome, Italy

with AOCA. Here, we present the clinical history and instrumental data of these three cases.

Materials and Methods

Between December 1986 and December 1996, at the Sports Medicine Laboratory of the Catholic University in Rome, 3,650 subjects (including 2,261 male and 1,389 female individuals, with a mean age of 30±12 years) practicing different sports at various competitive levels had a cardiologic examination for scientific or diagnostic purposes, including echocardiography. In Italy, athletes are required to undergo preparticipation screening. TTE was performed initially by one investigator (P.Z.), who has been trained in identifying the coronary ostia and proximal tracts of coronary arteries since 1985); since 1988, another investigator (C.S.) also has performed TTE. A commercially available mechanical sector scanner (Sonoline CD; Siemens Medical Systems, Inc; Iselin, NJ) equipped with a 3.5-MHz transducer was used. The coronary anatomy was first approached by the left parasternal short-axis aortic root view with mild inferosuperior or lateral angulation of the beam direction in order to obtain the best visualization of the ostia and initial tracts of the left (LCA) and right coronary artery (RCA). Technically satisfactory TTEs were obtained in 3,504 subjects (96%); the ostia and first tracts of both coronary arteries were clearly visualized in 3,150 cases (90%).

Minor variants of normal coronary arterial anatomy were identified in 56 cases (1.6%), and were considered normal variants. Variants included a separate origin of the left anterior descending artery and the left circumflex artery from the homologous sinus of Valsalva, or two distinct ostia in the right sinus for the right main coronary artery and for the conus branch.

Three out of 3,150 athletes (0.09%) were suspected to have an AOCA: an anomalous origin of the RCA from the left sinus in two cases, and an anomalous origin of the LCA from the right sinus in one. All three subjects underwent ECGs at rest, maximal ECG stress test, and 24-h ECG Holter monitoring, as well as cardiac MRI and myocardial scintigraphy with ^{99m}Tc-Sestamibi at rest and during exercise.

MRI scans were obtained on a whole-body imaging system (Vectra; General Electric Medical Systems; Milwaukee) operating at 0.5 T, and the patients were studied in the supine position. Multiple transverse and oblique images were obtained to visualize the proximal origin and path of the major epicardial coronary arteries, using a 4-mm slice thickness with 1-mm gap.

Single-photon emission computed tomographic myocardial scintigraphy was performed with IV administration of ^{99m}Tc-Sestamibi during exercise and at rest 24 h later.

After completing the abovementioned noninvasive procedures, the three athletes underwent cardiac catheterization, including coronary angiography and left ventriculography. Previous informed consent was obtained by all subjects extremely motivated to obtain a definitive diagnosis and evaluation in order to continue with their sports activity.

Case Reports

Case 1

A 17-year-old male basketball player was completely asymptomatic and had no family history of juvenile sudden death. During the first preparticipation medical examination for a competitive sport in November 1992, frequent ventricular ectopic beats were detected. The presence of polymorphous ventricular ectopic beats and couplets, mostly with a left branch block morphology, was confirmed with 24-h Holter monitoring. Stress ECG did not demonstrate any ST-T wave changes, and a TTE performed elsewhere was reported to be normal. In November 1994, the patient was referred to our laboratory because of the persistence of arrhythmias. His resting ECG was normal except for rare ventricular escape beats (Fig 1, *top*). TTE examination failed to identify the ostium of the RCA in the right sinus of Valsalva, but did reveal a normal origin from the left sinus and normal course of the LCA. An accurate exploration of the left sinus revealed an anomalous vessel, just in front of the LCA

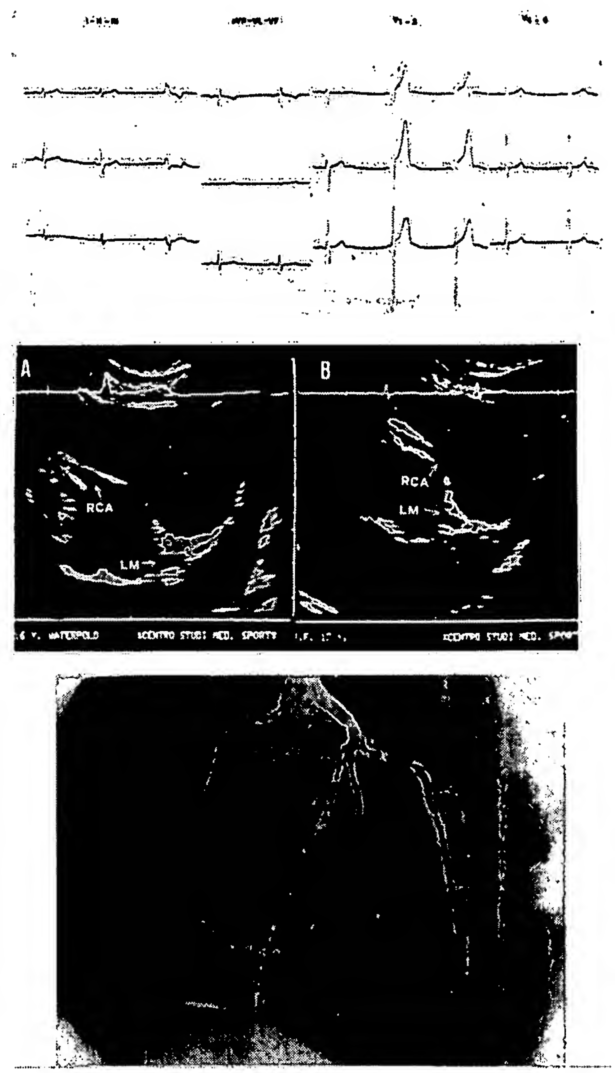


FIGURE 1. 17-year-old asymptomatic basketball player (case 1). *Top*, resting ECG was normal except for rare ventricular escape beats, single and coupled. *Center*, TTE (left parasternal short-axis aortic view). Panel B clearly shows the right coronary artery (RCA) originating abnormally from the left sinus of Valsalva, close to the left coronary artery ostium and the left main (LM), and running around between the aorta and pulmonary artery; for comparison, panel A shows the normal position of coronary ostia in a 16-year-old water polo player. *Bottom*, coronary angiography in the left oblique anterior view. Selective injection of contrast medium in the left sinus of Valsalva confirms that the right coronary artery originates from the left sinus just anterior to the left coronary ostium.

ostium, that originated obliquely, with a slit-like ostium, and ran between the aorta and pulmonary artery (Fig 1, *center*). The suspicion of an anomalous origin of the RCA from the left sinus of Valsalva was reinforced by MRI and definitively confirmed by coronary angiography (Fig 1, *bottom*). Exercise myocardial scintigraphy performed 1 week later failed to detect myocardial ischemia at maximal effort. The patient was advised to stop competitive sport activity and to undergo periodic examinations. One year later, Holter monitoring showed a clear reduction of the frequency and complexity of ventricular ectopic beats without a significant increase in average heart rate.

Case 2

An 18-year-old male collegiate middle-distance runner was completely asymptomatic and had no family history of juvenile sudden death. In January 1992, during a preparticipation medical examination for competitive sports, a resting ECG showed incomplete right bundle branch block associated with left-axis deviation. A TTE performed elsewhere was reportedly normal. Two years later, the runner was referred to us because of an episode of marked fatigue on effort during a soccer match and occasional episodes of dyspnea at rest. A maximal ECG stress test showed relative hypotension after exercise and accentuation of the left-axis deviation on ECG, but no arrhythmias or significant ST-T wave abnormalities. TTE examination revealed apparently normally contracting left and right ventricles. TTE and MRI exploration of the aortic root showed an anatomic picture quite similar to that observed in case 1, suggesting an anomalous origin of the RCA from the left sinus of Valsalva, which was confirmed by coronary angiography. Exercise myocardial scintigraphy revealed a mild reversible myocardial perfusion defect in the left ventricular apex. Coronary angiography failed to show a large posterior descending artery supplying the left ventricular apex, which was supplied mostly by the anterior descending artery. Therefore, the deficit in the apex should probably be considered a false-positive perfusion image. The athlete was advised to stop competitive sports activity and to undergo yearly examinations.

Case 3

A 47-year-old male long-distance runner participating in a master championship was completely asymptomatic and had no family history of juvenile sudden death. During his running career, he had completed several marathons. He had previously undergone some TTE examinations for a "cardiac murmur"; the TTEs were always described as normal. In January 1994, during the yearly medical examination for competitive athletes, his resting ECG was normal; the ECG stress test showed, for the first time, the appearance of left bundle branch block at a heart rate of more than 150 beats/min (Fig 2, *top right*). Exercise myocardial scintigraphy showed a reversible septal perfusion defect, which was attributed to left bundle branch block. Holter monitoring confirmed the presence of a rate-dependent left bundle branch block during training at a lower heart rate (120 beats/min). The patient was referred to us in February 1994. Another resting ECG was normal, except for marked sinus bradycardia. Routine TTE examination and MRI failed to identify the LCA ostium in its normal site but clearly demonstrated the presence of an anomalous vessel arising from the right (anterior) sinus of Valsalva and running with a posterior direction between the pulmonary artery and aortic root, suggesting an anomalous origin of LCA from the right sinus (Fig 2, *bottom left*

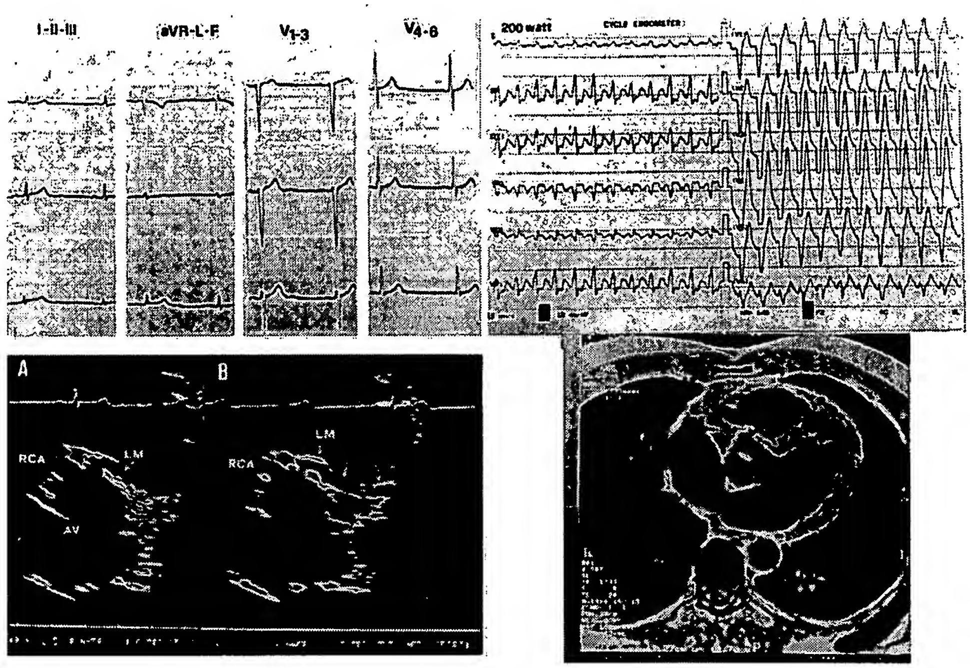


FIGURE 2. 47-year-old asymptomatic long-distance runner (case 3). *Top left*, resting ECG normal except for sinus bradycardia. *Top right*, maximal ECG stress test on bicycle ergometer showed the appearance of a complete left bundle branch block at a heart rate greater than 150 beats/min. *Bottom left*, TTE (left parasternal short-axis aortic view) revealed the absence of the left coronary ostium in the normal position and the presence of an anomalous vessel, the left coronary artery, arising from the right sinus of Valsalva; these anomalies were confirmed by MRI *(bottom right)* and coronary angiography (not shown).

and right), which was confirmed definitively by coronary angiography. The athlete was advised to refrain from strenuous physical activity.

Discussion and Conclusions

AOCA is a congenital anomaly that can be isolated or associated with other congenital cardiac diseases.[1,3] Different types of AOCA have been described in young adults, including the origin of a coronary artery directly from the pulmonary trunk, usually strongly symptomatic in early infancy, and the anomalous origin of the RCA and LCA from the opposite sinus of Valsalva. Four types of anomalous origin of the LCA were described by Roberts,[1] type B being the most frequent in autopsies of subjects with sudden death or death attributed to ischemic causes. In this type, the LCA arises from the right sinus of Valsalva at an angle of roughly 180° to the center of aorta, has a slit-like ostium, and runs between the pulmonary trunk and the aorta. In the anomalous origin of the RCA from the left sinus, the anatomic picture is similar but the ostium is less frequently slit-like.[1]

The prevalence of AOCA in the general population is very low. Recently, Pelliccia et al[9] did not find any cases of AOCA in a large cohort of élite athletes. However, in spite of its rarity, sports medicine physicians should not disregard AOCA because this problem may be associated with sudden death in young people, particularly those participating in sports or performing strenuous exercise.[4-6,11] The association of AOCA with exercise-related sudden death has been explained by three factors: (1) outward expansion of the roots of both the aorta and pulmonary trunk during exertion, which can cause further compression of the ostial lumen of the anomalous artery; (2) the anomalous vessel can be compressed against the root of the pulmonary trunk, where it is firmly anchored to the infundibular septum, when the aortic root and pulmonary trunk dilate during exertion; and (3) myocardial oxygen requirement increases with exertion.[1] The consequent myocardial ischemia may trigger life-threatening ventricular arrhythmias and sudden death.

Unfortunately, AOCA is a very insidious pathology, difficult to identify *in vivo* because the affected subjects are often asymptomatic and sudden death can be the first manifestation of the disease. Only 30% of symptomatic patients had typical angina,[4] while the remaining ones reported nonspecific symptoms such as syncope, dyspnea on effort, or ventricular arrhythmias. TTE examination may be useful in identifying AOCA. However, sonographers need specific training and a high-quality imaging system to visualize the origins of coronary arteries. This may explain why all three subjects in our study had prior TTEs that failed to identify the anomaly.

Today, transesophageal echocardiography has greatly improved the likelihood of diagnosing AOCA and identifying an abnormal course between the aorta and pulmonary trunk, which sometimes cannot be visualized satisfactorily even by coronary angiography.[12,13] As demonstrated by our cases, MRI also appeared to be a promising tool for identifying such anomalies.[14-16] However, in our cases, MRI showed a complementary role, and transesophageal echocardiography was not performed because the traditional TTE permitted successful identification of these anomalies.

Nevertheless, our cases confirm the difficulty of making an *in vivo* diagnosis of AOCA in athletes. The three patients did not have angina at rest or on effort, nor did they have other cardiovascular symptoms, such as syncope, dyspnea, or palpitations. A resting ECG was practically normal in cases 1 and 3, while in case 2 it showed only incomplete right bundle branch block with left-axis-deviation. Stress ECGs revealed abnormalities in all three cases, but none was specific for exercise-induced ischemia: the patient in case 1 had ventricular ectopic beats; case 2, an accentuation of left-axis deviation; and case 3, a rate-dependent left bundle branch block. Furthermore, the patient in case 1 had negative myocardial scintigraphy; case 2 had a mild reversible apical defect; and case 3 had a reversible septal perfusion defect, which was attributed, however, to left bundle branch block. Therefore, ECG stress testing and myocardial scintigraphy may provide little or no diagnostic information in patients with suspected AOCA.[1]

Although asymptomatic, all three athletes were advised to refrain from competitive sports and strenuous physical activity because of the increased risk of exercise-related sudden death, and careful follow-up was recommended. Little is known about the natural history of these anomalies, particularly when the subjects are no longer competitive athletes.

In conclusion, we found three cases of AOCA in a large cohort of athletes playing different sports at various competitive levels; the prevalence of 0.09% was similar to that reported for the general population.[1,17] This apparently contrasts with the complete absence of AOCA reported by Pelliccia et al.[9] However, the prevalence of AOCA in our cohort may be higher since some subjects (including all three cases) were referred for evaluation of cardiac problems or symptoms.

Our study confirmed that AOCA is rare in asymptomatic athletes, but that it can be identified through an accurate exploration of coronary anatomy by TTE. This should not imply that a routine TTE

should be included in the preparticipation screening of athletes since the cost-effectiveness ratio of this procedure is unfavorable.[18] For this reason, also in our country, where a preparticipation screening of athletes is required by law, so directly implicating responsibility of sports physicians in the eligibility for competitive sports, we do not recommend to include it. We would simply alert sports physicians and cardiologists to the utility of accurate TTE to exclude or confirm the presence of an AOCA in athletes referred for unexplained symptoms and/or ECG abnormalities.

ACKNOWLEDGMENTS: The authors thank Professor Gaetano Thiene from the Human Pathology Institute of Padova University for his revision of the text and valuable advice.

References

1 Roberts WC. Major anomalies of coronary arterial origin seen in adulthood. Am Heart J 1986; 111:941-63
2 Hobbs RE, Millit HD, Raghavan PV, et al. Congenital coronary artery anomalies: clinical and therapeutic implications. Cardiovasc Clin 1981; 12:43-58
3 Taylor AJ, Rogan KM, Virmani R. Sudden cardiac death associated with isolated congenital coronary artery anomalies. J Am Coll Cardiol 1992; 20:640-47
4 Liberthson RR. Sudden death from cardiac causes in children and young adults. N Engl J Med 1996; 334:1039-44
5 Maron BJ, Roberts WC, McAllister MA, et al. Sudden death in young athletes. Circulation 1980; 62:218-29
6 Corrado D, Thiene G, Cocco P, et al. Non-sclerotic coronary artery disease and sudden death in the young. Br Heart J 1992; 68:601-07
7 Weyiman AE, Feigenbaum H, Dillon JC, et al. Noninvasive visualization of the left main coronary artery by cross-sectional echocardiography. Circulation 1976; 54:169-74
8 Zeppilli P, Rubino P, Manno V, et al. Visualizzazione ecocardiografica delle arterie coronarie in atleti di resistenza. G Ital Cardiol 1987; 17:957-65
9 Pelliccia A, Spataro A, Maron BJ. Prospective echocardiographic screening for coronary artery anomalies in 1360 elite competitive athletes. Am J Cardiol 1993; 72:978-79
10 Zeppilli P. The athlete's heart: differentiation of training effects from organic heart disease. Pract Cardiol 1988; 14(8): 61-84
11 Burke AP, Farb A, Virmani R, et al. Sports-related and non-sports-related sudden cardiac death in young adults. Am Heart J 1991; 121:568-75
12 Fernandez F, Alam M, Smith S, et al. The role of transesophageal echocardiography in identifying anomalous coronary arteries. Circulation 1993; 88:2532-36
13 Gaither NS, Rogan KM, Stajduhar K, et al. Anomalous origin and course of coronary arteries in adults: identification and improved imaging utilizing transesophageal echocardiography. Am Heart J 1991; 122:69-75
14 Machado C, Bhasin S, Soulen RF. Confirmation of anomalous origin of the right coronary artery from the left sinus of Valsalva with magnetic resonance imaging. Chest 1993; 104: 1284-86
15 McConnell MV, Ganz P, Selwyn AP, et al. Identification of anomalous coronary arteries and their anatomic course by magnetic resonance coronary angiography. Circulation 1995; 92:3158-62
16 Post JC, van Rossum AC, Bronzwaer JGF, et al. Magnetic resonance angiography of anomalous coronary arteries: a new gold standard for delineating the proximal course? Circulation 1995; 92:3163-71
17 Topaz EJ, De Marchena EP, Sommer LS, et al. Anomalous coronary arteries: angiographic findings in 80 patients. Int J Cardiol 1992; 34:129-38
18 Maron BJ, Thompson PD, Puffer JC, et al. Cardiovascular preparticipation screening of competitive athletes. Circulation 1996; 94:850-56

C

CA CANCER J CLIN 1997;47:154-160

American Cancer Society Guidelines for Screening and Surveillance for Early Detection of Colorectal Polyps and Cancer: Update 1997

Tim Byers, MD, MPH; Bernard Levin, MD; David Rothenberger, MD; Gerald D. Dodd, MD; and Robert A. Smith, PhD (for the American Cancer Society Detection and Treatment Advisory Group on Colorectal Cancer)

Background

In 1997 approximately 131,000 Americans will be diagnosed with colorectal cancer and about 55,000 will die from this disease.[1] Colorectal cancer is second only to lung cancer as a cause of death from cancer in the United States. Without preventive actions, about 6% of Americans will develop colorectal cancer sometime in their lives. Recent research, however, has contributed to a growing consensus that early detection methods can prevent a substantial proportion of the suffering and mortality from colorectal cancer.[2,3] The same methods used to detect colorectal cancers at early, curable stages can also aid physicians in the identification and removal of adenomas, which give rise to colorectal cancer. Methods for early detection can therefore actually prevent colorectal cancer.

Dr. Byers is Professor of Preventive Medicine at the University of Colorado School of Medicine in Denver, Colorado.

Dr. Levin is Vice President for Cancer Prevention and Professor of Medicine at the University of Texas M.D. Anderson Cancer Center in Houston, Texas.

Dr. Rothenberger is Clinical Professor and Chief of the Division of Colon and Rectal Surgery at the University of Minnesota in Minneapolis, Minnesota.

Dr. Dodd is Emeritus Professor of Radiology at the University of Texas M.D. Anderson Cancer Center in Houston, Texas.

Dr. Smith is Senior Director for Cancer Detection and Treatment for the American Cancer Society in Atlanta, Georgia.

The ACS Detection and Treatment Advisory Group on Colorectal Cancer consisted of the following members: Bernard Levin, MD (Chair);* Tim Byers, MD, MPH (Vice-Chair);* Donald Anthony, MD; Debra Broadwell-Jackson, PhD, RN; Randall Burt, MD; Jerome DeCosse, MD; Gerald Dodd, MD;* Julian Duttera, MD; John Fazekas, MD; Cecilia Fenoglio-Preiser, MD; Stanley Hamilton, MD; Edward Mansour, MD; Richard Nelson, MD; Jerry Olshan, DO; Michael Paglia, MD; Mary Elizabeth Roth, MD; David Rothenberger, MD;* Robert Schweitzer, MD; Mary Simmonds, MD; Marion Nadel, PhD (CDC liaison); Robert Smith, PhD (ACS staff).*

*Screening guidelines working group.

Development of Guidelines

The American Cancer Society issues guidelines for practitioners and the general public for both clinical practices and lifestyle behaviors that can reduce suffering and mortality from cancer. Since 1980 the ACS has recommended screening for colorectal cancer.[4] The current recommendation, updated in 1992, calls for everyone over age 50 who is at average risk to be screened with annual fecal occult blood testing and sigmoidoscopy every 3 to 5 years and for those at higher risk to seek a recommendation from their physician.[5]

Because of new findings that have strengthened the evidence of benefit from screening people at average risk and

because of the need for more specific guidance for those at higher risk, the ACS has determined that these guidelines now need to be updated.

The ACS Detection and Treatment Advisory Group on Colorectal Cancer developed the new guidelines using an inclusive process, based on the best scientific evidence available. Late in 1995, the Advisory Group met to review the scientific evidence and to set general principles for guideline development. The Group identified four scientific and clinical issues that it regarded as important in the formation of these new guidelines:

1. Evidence was growing about the benefits of early detection of colorectal cancer and of adenomatous polyp removal.

2. Evidence was inconclusive regarding the relative clinical effectiveness of the alternative methods for early detection of colorectal cancer and adenomatous polyps.

3. Advances in cancer genetics and findings from epidemiologic studies had identified increasing numbers of people as being at high risk for colorectal cancer, yet the previous ACS guidelines did not provide specific recommendations for anyone other than those at average risk.[4]

4. Despite the existing ACS guidelines most adults in the United States were not being screened for colorectal cancer.

The Advisory Group developed the new guidelines by a process based on the following four principles:

1. Wide input and consensus among the medical specialties would be sought.

2. Specific advice would be offered when scientific data could support specific methods, and a range of acceptable options would be offered when scientific data were insufficient to contrast alternative effective methods.

3. Different recommendations would be considered for those at different levels of risk.

4. The guidelines would be clear enough for widespread clinical use but also flexible enough to allow for tailoring of approaches to meet individual needs.

Draft guidelines were developed in mid-1996 and circulated to various organizations for comments and suggestions. Based on those suggestions, the guidelines were then revised in early 1997 and approved by a vote of the ACS Board of Directors in March 1997.

Definitions of Early Detection Methods

Fecal occult blood testing (FOBT) refers to the implementation of the protocol of collecting and testing six samples from three consecutive stools of a patient following a specified diet.[6] The published FOBT trials used the Hemoccult II method (SmithKline Diagnostics, San Jose, CA),[7-9] but other methods are now in development.[10] A positive fecal occult blood test should be followed by colonoscopy.

Digital rectal examination refers to the inspection and palpation of the anus and lower rectum.

Flexible sigmoidoscopy refers to the direct visual examination of the lower third to half of the colorectum by a trained examiner using a flexible 60-cm endoscope after satisfactory cleansing of the descending and sigmoid colon. A positive finding on sigmoidoscopy should be followed by colonoscopy.

Total colon examination refers to either of two procedures carried out by a trained examiner after a satisfactory cleansing of the entire colorectum. One procedure is *colonoscopy*, direct visual examination of the entire colorectum (to the cecum) using a colonoscope. The other procedure is *double-contrast barium enema*, radiologic examination of the entire colorectum by instilling both barium and air to define the contours of the colorectal mucosa. A positive finding on double-contrast barium enema should usually be followed by endoscopy.

1997 ACS Colorectal Cancer Screening Guidelines

The new ACS guidelines for the early detection of colorectal adenomas and colorectal cancer are summarized in the Table. Following is a brief explanation of the main features of the recommendations.

People at Average Risk

Approximately 70 to 80% of all colorectal cancers occur among people at "average risk."[3] Average risk is defined by exclusion as anyone who is not otherwise defined as being at increased risk (as defined below). There are no factors yet identified that would place a person at lower than "average" risk for an initial approach to screening.

Everyone should begin colorectal cancer screening by age 50 by one of two methods, annual FOBT with sigmoidoscopy every 5 years or a total colon examination, either by colonoscopy (every 10 years) or by double-contrast barium enema (every 5 to 10 years). Digital rectal examination should be performed at the time of the sigmoidoscopy or the total colon examination, that is, every 5 to 10 years. Although there may be benefits of more frequent digital rectal examination for other purposes (e.g., for examination of the prostate in men or as part of the bimanual pelvic examination in women), there is no added benefit in detecting anal or rectal neoplasia by digital rectal examination more often than every 5 to 10 years.

The choice of colonoscopy or double-contrast barium enema can be made on an individual basis, depending on factors such as the local availability of trained clinicians who can offer a high-quality examination and cost. A supplementary double-contrast barium enema may be needed if a colonoscopic examination fails to reach the cecum, and a supplementary colonoscopy may be needed if a double-contrast barium enema identifies a possible lesion or does not adequately visualize the rectosigmoid area. Double-contrast barium enema is usually less expensive than colonoscopy, but colonoscopy provides a direct visualization of lesions, which can then be sampled for biopsy or excised during the same procedure. For those who elect a periodic total colon examination for screening, there is no need for annual FOBT.

People at Moderate Risk

Approximately 15 to 20% of colorectal cancers occur among people at moderate risk.[3] The genetic events leading to colorectal cancer are rapidly becoming understood.[11] Colorectal adenomas are clearly the precursor lesions for almost all colorectal cancers, and adenomas are usually present for several years before they develop into cancer.[12] People who are diagnosed as having adenomatous polyps should have colonoscopic removal of all polyps from the colorectum. A total colon examination should then be repeated in 3 years. If adenomatous polyps have not recurred at the time of the 3-year examination, surveillance by total colon examination should be repeated every 5 years thereafter if the original polyp was 1 cm or larger or contained villous histology. If the original polyp was smaller than 1 cm, however, if it did not contain villous histology, and if the 3-year examination is negative, the patient can thereafter return to "average-risk" recommendations.

A family history of either colorectal cancer or colorectal adenomas increases the risk of developing colorectal cancer. In general, the closer the familial relationship, the younger the age of onset, and the larger the number of affected family members, the greater is the risk.[13] Thus, risk is especially high for an individual if a first-degree relative (parent, sibling, or offspring) has had a colorectal

ACS Guidelines for Screening and Surveillance for Early Detection of Colorectal Polyps and Cancer*

Risk Category	Recommendation†	Age to Begin	Interval
AVERAGE RISK			
All people 50 years or older who are not in the categories below	One of the following: FOBT plus flexible sigmoidoscopy‡ or TCE¶	Age 50 Age 50	FOBT every year and flexible sigmoidoscopy every 5 y Colonoscopy every 10 y or DCBE every 5–10 y
MODERATE RISK			
People with single, small (<1 cm) adenomatous polyps	Colonoscopy	At time of initial polyp diagnosis	TCE within 3 y after initial polyp removal; if normal, as per average risk recommendations (above)
People with large (≥1 cm) or multiple adenomatous polyps of any size	Colonoscopy	At time of initial polyp diagnosis	TCE within 3 y after initial polyp removal; if normal, TCE every 5 y
Personal history of curative-intent resection of colorectal cancer	TCE§	Within 1 y after resection	If normal, TCE in 3 y; if still normal, TCE every 5 y
Colorectal cancer or adenomatous polyps in first-degree relative younger than 60 y or in two or more first-degree relatives of any ages	TCE	Age 40 or 10 y before the youngest case in the family, whichever is earlier	Every 5 y
Colorectal cancer in other relatives (not included above)	As per average risk recommendations (above); may consider beginning screening before age 50		
HIGH RISK			
Family history of familial adenomatous polyposis	Early surveillance with endoscopy, counseling to consider genetic testing, and referral to a specialty center	Puberty	If genetic test positive or polyposis confirmed, consider colectomy; otherwise, endoscopy every 1–2 y
Family history of hereditary non-polyposis colon cancer	Colonoscopy and counseling to consider genetic testing	Age 21	If genetic test positive or if patient has not had genetic testing, colonoscopy every 2 y until age 40 y, then every year
Inflammatory bowel disease	Colonoscopies with biopsies for dysplasia	8 y after the start of pancolitis; 12–15 y after the start of left-sided colitis	Every 1–2 y

*Approximately 70–80% of cases are from average-risk individuals, approximately 15–20% are from moderate-risk individuals, and 5–10% are from high-risk individuals.

†Digital rectal examination should be done at the time of each sigmoidoscopy, colonoscopy, or DCBE.

‡Annual FOBT has been shown to reduce mortality from colorectal cancer, so it is preferable to no screening; however, the ACS recommends that annual FOBT be accompanied by flexible sigmoidoscopy to further reduce the risk of colorectal cancer mortality.

¶TCE includes either colonoscopy or DCBE. The choice of procedure should depend on the medical status of the patient and the relative quality of the medical examinations available in a specific community. Flexible sigmoidoscopy should be performed in those instances in which the rectosigmoid colon is not well visualized by DCBE. DCBE would be performed when the entire colon has not been adequately evaluated by colonoscopy.

§This assumes that a perioperative TCE was done.

DCBE = double-contrast barium enema; FOBT = fecal occult blood testing; TCE = total colon examination; y = years.

cancer or an adenomatous polyp diagnosed before age 60 years or if more than one first-degree relative has been affected at any age. Individuals with a single first-degree relative diagnosed with a colorectal cancer or an adenomatous polyp after age 60, however, or with affected relatives who are not first-degree relations can be considered to be at average risk, although it may be prudent to begin screening before the age of 50 years.

People at High Risk

Approximately 5 to 10% of all colorectal cancers occur among people at high risk.[3] Most of those defined as being at high risk for colorectal cancer have one of two hereditary syndromes or inflammatory bowel disease.

Familial adenomatous polyposis syndrome (FAP) is a genetic condition that affects 1 in 10,000 people. FAP is caused by a mutation in the APC gene on chromosome 5.[11] People with this condition develop hundreds of colorectal polyps and will almost certainly develop colorectal cancer unless the colon is removed.

Hereditary non-polyposis colorectal cancer syndrome (HNPCC) is a genetic condition that can also cause colorectal cancer among many people in a family even though multiple polyps are not present. HNPCC is caused by mutations in mismatch repair genes located on chromosome 2, 3, or 7.[11] HNPCC has been classically defined as colorectal cancer in three or more family members, two of whom are first-degree relatives of the third, involving people in at least two generations, and with one person diagnosed with colorectal cancer before age 50 years. However, other variants of this classic pedigree clearly exist. Individuals with genetic mutations that can lead to HNPCC are also at high risk for cancers of the ovary, uterus, ureter, pancreas, and stomach. Genetic tests are now available to detect the mutations that lead to FAP and HNPCC and should be considered, with appropriate counseling, for people with family histories suggestive of these conditions.

Both ulcerative colitis and Crohn's disease that affects the colorectum greatly increase risk for colorectal cancer beginning 8 years after the onset of colorectal symptoms. Management is by careful endoscopic surveillance for colonic dysplasia and prophylactic colectomy.

Comment

These new ACS guidelines for colorectal cancer screening are comparable to guidelines released by two other organizations within the past year.[2,3]

In 1996 the US Preventive Services Task Force (USPSTF) issued revised guidelines for clinical preventive services.[2] Those guidelines called for annual FOBT beginning at age 50. The success of a large randomized, controlled trial in reducing colorectal cancer mortality by 33% was the basis of the USPSTF recommendation.[7] Since then, two European trials also have shown benefits from FOBT.[8,9] The USPSTF also recommended sigmoidoscopic screening, though it did not specify a frequency because no randomized trials testing sigmoidoscopy had yet been completed.[2] The USPSTF criteria for making recommendations depend largely on findings from randomized trials. A randomized, controlled trial is now under way to measure the benefits of sigmoidoscopic screening, but results will not be known for several years.[14]

The ACS recommends that flexible sigmoidoscopy be done every 5 years to complement annual FOBT. One reason for the ACS recommendation is related to a well-documented set of recommendations issued in February 1997 by an interdisciplinary task force originally convened by the Agency for Health Care Policy Research (AHCPR).[3] This Task Force was supported not only by AHCPR but also by the American Gastroenterological Association, the Ameri-

can Society for Gastrointestinal Endoscopy, the American College of Gastroenterology, the American Society of Colon and Rectal Surgeons, and the Society of American Gastroenterologic Endoscopic Surgeons. The Task Force called for universal screening for everyone after age 50 years, offered a set of reasonable options for screening methods, and specified recommendations for different levels of risk. These new ACS guidelines for colorectal cancer screening are nearly identical to the AHCPR Task Force guidelines.

One of the Task Force options is limited to annual FOBT testing for those at average risk for colorectal cancer. The ACS recommendation to always add periodic sigmoidoscopic examinations to the annual FOBT is an important difference between the ACS and the AHCPR Task Force recommendations. The ACS has made this recommendation for three reasons: the benefits from the FOBT trials[7-9] were the result of the diagnostic endoscopies that followed positive FOBT tests,[15] other studies have shown substantial risk reduction from sigmoidoscopic examinations,[16,17] and flexible sigmoidoscopy is now widely available and safe and can be offered at a reasonable cost. Although trials have shown that FOBT alone as a screening method can reduce colorectal cancer mortality by 15 to 33%,[7-9] the benefits derived from FOBT in these trials were the result not of the FOBT per se but of the subsequent colonoscopic examinations that led to polypectomy or to the early identification of colorectal cancers.[15] The simulation models presented by the AHCPR Task Force estimate a 20% reduction in mortality from adding sigmoidoscopy every 5 years to annual FOBT,[3] and two case-control studies suggest an even greater benefit from sigmoidoscopy.[16,17] The ACS therefore recommends sigmoidoscopic examinations every 5 years as a reasonable way to intensively monitor the left side of the colorectum for neoplasia while using annual FOBT to monitor the entire colon for bleeding.

The option of using total colon examination as a primary screening method is an important feature of both the AHCPR Task Force recommendations and the new ACS guidelines. Although randomized trials of total colonic examinations have not yet begun (and hence measures of their effectiveness will not be made for at least 10 years), the ACS committee believes that the indirect evidence for benefit and efficiency is compelling and that both benefit and efficacy would probably improve if future costs declined and availabilities improved with greater demand.

The ACS committee discussed the operational performance of various age cut-points. Because cut-points for age are arbitrary in most publications, the ACS committee decided to recommend a common age of "before age 60 years" to define familial risk. This is the same as the AHCPR Task Force cut-point for polyps but 5 years higher (and thus more inclusive) than the Task Force cut-point for cancer. Minor differences in the cut-points are less important than the concept of identifying elevated risk by family history, then beginning screening at an earlier age.

Future developments will improve the already impressive benefits of the early detection methods we now have in place in many clinical centers. FOBT methods will probably improve and be refined. The clinical implications of rehydration of Hemoccult II slides (resulting in high sensitivity but lower specificity) have resulted in an appreciation of the need for newer tests, such as Hemoccult Sensa (SmithKline Diagnostics, San Jose, CA) and immunochemical tests for human hemoglobin. The use of different types of fecal occult blood tests in combination is also under study. Even genetic testing for mutations present in colonic cells excreted in feces is technically possible,[18] and improved imaging techniques

using modalities such as spiral CT with three-dimensional reconstruction of the colonic images may be useful for early detection.[19] These new ACS guidelines for screening and surveillance for the early detection of colorectal polyps and cancer should be useful for clinicians and the general public. These guidelines will be updated in the future as new scientific data become available.

Summary

In the past, differences in opinion among professional groups about colorectal cancer screening have been a barrier to colorectal cancer prevention. It is clear that screening for colorectal cancer is currently practiced by fewer than 20% of American adults.[20] However, a growing consensus now exists that even though we do not yet have trial data to compare precisely the various methods for screening, there is now both a compelling case for screening and a reasonable set of methods that clinicians and patients can consider. By applying the knowledge we already have, it is likely that most of the deaths from colorectal cancer in the United States could be prevented.

References

1. Parker S, Tong T, Bolden S, et al: Cancer statistics, 1997. CA Cancer J Clin 1997;47:5-27.
2. U.S. Preventive Services Task Force: Guide to Clinical Preventive Services: Report of the U.S. Preventive Services Task Force, ed 2. Washington, DC, Government Printing Office, 1996.
3. Winawer SJ, Fletcher RH, Miller L, et al: Colorectal cancer screening: Clinical guidelines and rationale. Gastroenterology 1997;112:594-642.
4. Eddy D: Guidelines for the cancer-related checkup: Recommendations and rationale. CA Cancer J Clin 1980;30:3-50.
5. Levin B, Murphy G: Revision in American Cancer Society recommendations for the early detection of colorectal cancer. CA Cancer J Clin 1992;42:296-299.
6. Young GP, Macrae FA, St. John DJ: Clinical methods for early detection: Basis, use and evaluation, in Young GP, Rozen P, Levin B (eds): Prevention and Early Detection of Colorectal Cancer. London, W.B. Saunders, 1996, pp 241-270.
7. Mandel JS, Bond JH, Church TR, et al: Reducing mortality from colorectal cancer by screening for fecal occult blood: Minnesota Colon Cancer Control Study. N Engl J Med 1993;328:1365-1371.
8. Kronborg O, Fenger C, Olsen J, et al: Randomised study of screening for colorectal cancer with faecal-occult-blood test. Lancet 1996;348:1467-1471.
9. Hardcastle JD, Chamberlain JO, Robinson MH, et al: Randomised controlled trial of faecal-occult-blood screening for colorectal cancer. Lancet 1996;348:1472-1477.
10. Allison JE, Tekawa IS, Ransom LJ, et al: A comparison of fecal occult-blood tests for colorectal-cancer screening. N Engl J Med 1996;334:155-159.
11. Kinzler KW, Vogelstein B: Lessons from hereditary colorectal cancer. Cell 1996;87:159-170.
12. Winawer SJ, Zauber AG, Ho MN, et al: Prevention of colorectal cancer by colonoscopic polypectomy: The National Polyp Study Workgroup. N Engl J Med 1993;329:1977-1981.
13. Winawer SJ, Zauber AG, Gerdes H, et al: Risk of colorectal cancer in the families of patients with adenomatous polyps: National Polyp Study Workgroup. N Engl J Med 1996;334:82-87.
14. Gohagan JK, Prorok PC, Kramer BS, et al: The prostate, lung, colorectal and ovarian cancer screening trial of the National Cancer Institute. Cancer 1995;75:1869-1873.
15. Lang CA, Ransohoff DF: Fecal occult blood screening for colorectal cancer: Is mortality reduced by chance selection for screening colonoscopy? JAMA 1994;271:1011-1013.
16. Selby JV, Friedman GD, Quesenberry CP Jr, et al: A case-control study of screening sigmoidoscopy and mortality from colorectal cancer. N Engl J Med 1992;326:653-657.
17. Newcomb PA, Norfleet RG, Storer BE, et al: Screening sigmoidoscopy and colorectal cancer mortality. J Natl Cancer Inst 1992;84:1572-1475.
18. Sidransky D, Tokino T, Hamilton S, et al: Identification of *ras* oncogene mutations in the stool of patients with curable colorectal tumors. Science 1992;256:102-105.
19. Hara A, Johnson C, Reed J, et al: Detection of coloretal polyps by computed tomographic colography: Feasibility of a novel technique. Gastroenterology 1996;110:284-290.
20. Trends in cancer screening—United States, 1987 and 1992. MMWR Morb Mortal Wkly Rep 1996;45:57-61.

Screening for type 2 diabetes
Anonymous
Diabetes Care; Jan 1999; 22, Research Library
pg. S20

POSITION STATEMENT

Screening for Type 2 Diabetes

AMERICAN DIABETES ASSOCIATION

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Type 2 diabetes, the most prevalent form of the disease, is often asymptomatic in its early stages and can remain undiagnosed for many years. Approximately 5.4 million adults in the U.S. have undiagnosed type 2 diabetes.

The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Individuals with undiagnosed type 2 diabetes are at significantly higher risk for coronary heart disease, stroke, and peripheral vascular disease than the nondiabetic population. They also have a greater likelihood of having dyslipidemia, hypertension, and obesity.

Because early detection and prompt treatment may reduce the burden of type 2 diabetes and its complications, screening for diabetes may be appropriate under certain circumstances. This position statement provides recommendations for diabetes screenings performed in physicians' offices and community screening programs.

This position statement does not address screening for type 1 diabetes or gestational diabetes mellitus (GDM). Because of the acute onset of symptoms, most cases of type 1 diabetes are detected soon after symptoms develop. Widespread clinical testing of asymptomatic individuals for the presence of autoantibodies related to type 1 diabetes cannot be recommended at this time as the means to identify persons at risk. Reasons for this include the following: *1*) cutoff values for some of the immune marker assays have not been completely established for clinical settings; *2*) there is no consensus as to what action should be taken when a positive autoantibody test result is obtained; and *3*) because the incidence of type 1 diabetes is low, testing of healthy children will identify only the small number (<0.5%) who at that moment may be "prediabetic." Clinical studies are being conducted to test various methods of preventing type 1 diabetes in high-risk subjects (e.g., siblings of type 1 patients). These studies may uncover an effective means of preventing type 1 diabetes, in which case screening may be appropriate in the future.

For information on screening for GDM, refer to the American Diabetes Association's position statement "Gestational Diabetes Mellitus."

DIABETES PREVALENCE AND RISK FACTORS — The prevalence of diagnosed and undiagnosed diabetes in adults in the U.S. is about 6%. However, specific subgroups have a much higher prevalence of the disease than the population as a whole. These subgroups have certain attributes or risk factors that either directly cause diabetes or are statistically associated with it.

The correlation of a risk factor(s) with development of diabetes is never 100%. However, the greater the number of risk factors present in an individual, the greater the chance of that individual developing or having diabetes. Conversely, the chance of finding diabetes in an individual without a risk factor is low. Thus, the likelihood of identifying an asymptomatic individual with diabetes in the general population through random screening is small; however, in high-risk groups the likelihood is much greater.

The risk of developing type 2 diabetes increases with age, obesity, and lack of physical activity. Type 2 diabetes is more common in individuals with a family history of the disease and in members of certain racial/ethnic groups. It occurs more frequently in women with prior GDM and in individuals with hypertension or dyslipidemia. Major risk factors for type 2 diabetes are summarized in Table 1.

SYMPTOMS — Symptoms of marked hyperglycemia include polyuria; polydipsia; weight loss, sometimes with polyphagia; and blurred vision. However, each of these symptoms are nonspecific and, therefore, can occur in individuals without diabetes. Thus, in and of themselves, symptoms are poor indicators of diabetes. Also, type 2 diabetes frequently goes undiagnosed for many years because the hyperglycemia develops gradually, and thus the onset, and even the presence, of symptoms often goes unnoticed.

GENERAL RECOMMENDATIONS FOR SCREENING BY PHYSICIANS — Screening for diabetes as part of routine medical care may be appropriate if the patient has one or more of the risk factors shown in Table 1. Based on the lack of high-quality cost-benefit studies, it is premature to recommend screening all high-risk individuals. Thus, the decision to screen for diabetes should ultimately be based on clinical judgment and patient preference.

On the basis of expert opinion, screening of high-risk individuals should be considered at 3-year intervals. The rationale for this interval is that there is little likelihood of an individual developing any of the complications of diabetes to a significant degree within 3 years of a negative screening test result.

Screening tests

The fasting plasma glucose (FPG) test and the oral glucose tolerance test (OGTT) are both suitable tests for diabetes; however, the FPG test is strongly preferred because it is easier and faster to perform, more convenient and acceptable to patients, and less expensive. Fasting is defined as no consumption of food or beverage other than water for at least 8 h before testing.

An FPG level ≥126 mg/dl (7.0 mmol/l) or a 2-h postload value in the OGTT ≥200 mg/dl (11.1 mmol/l) are indications for retesting. Either test must be repeated on a different day to confirm a diagnosis. Table 2 presents the diagnostic criteria.

Originally approved April 1989. Most recent review/revision 1998.

Abbreviations: FPG, fasting plasma glucose; GDM, gestational diabetes mellitus; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; OGTT, oral glucose tolerance test.

Table 1—*Major risk factors for diabetes mellitus*

Family history of diabetes (i.e., parents or siblings with diabetes)
Obesity (i.e., ≥20% over desired body weight or BMI ≥27 kg/m²)
Race/ethnicity (e.g., African-Americans, Hispanic-Americans, Native Americans, Asian-Americans, Pacific Islanders)
Age ≥45 years
Previously identified IFG or IGT
Hypertension (≥140/90 mmHg)
HDL cholesterol level ≤35 mg/dl (0.90 mmol/l) and/or a triglyceride level ≥250 mg/dl (2.82 mmol/l)
History of GDM or delivery of babies over 9 lb

Nondiabetic individuals with an FPG ≥110 mg/dl (6.1 mmol/l) but <126 mg/dl (7.0 mmol/l) are considered to have impaired fasting glucose (IFG), and those with 2-h values in the OGTT ≥140 mg/dl (7.8 mmol/l) but <200 mg/dl (11.1 mmol/l) are defined as having impaired glucose tolerance (IGT). Both IFG and IGT are risk factors for future diabetes.

Normoglycemia is defined as plasma glucose levels <110 mg/dl (6.1 mmol/l) in the FPG and a 2-h postload value <140 mg/dl (7.8 mmol/l) in the OGTT.

If necessary, plasma glucose testing may be performed on individuals who have taken food or drink shortly before testing. Such tests are referred to as random plasma glucose measurements and are given without regard to time of last meal. A random plasma glucose level ≥160 mg/dl (8.9 mmol/l) is considered a positive screening test result. The diagnosis of diabetes is made on additional testing—preferably by the FPG test, or less preferably, by the OGTT—performed on subsequent days.

Laboratory measurement of plasma glucose concentration is performed on venous samples with enzymatic assay techniques, and the above-mentioned values are based on the use of such methods.

Glycated hemoglobin remains a valuable tool for monitoring glycemia, but it is not currently recommended for the screening or diagnosis of diabetes.

Other considerations

In screening for disease, it is crucial that appropriate interpretation of the screening test results is provided to the patient and that follow-up evaluation and treatment are made available. Also, it is important to consider that certain drugs, including glucocorticoids, furosemide, thiazides, estrogen-containing products, β-blockers, and nicotinic acid, may produce hyperglycemia.

GENERAL RECOMMENDATIONS FOR COMMUNITY SCREENING PROGRAMS

— The major objective of a community screening program is to identify individuals at high risk for having undiagnosed diabetes. To this end, a verbal or written questionnaire is recommended. The American Diabetes Association's diabetes risk test is shown in Fig. 1. Individuals who have been determined to be at risk should be referred to a physician for evaluation. In addition, individuals indicating that they have any diabetes symptoms should seek appropriate medical care. Community screening programs must have an established mechanism for referring individuals to a clinician for further evaluation.

Although there is ample scientific evidence showing that certain risk factors predispose individuals to development of diabetes (Table 1), there is insufficient evidence to conclude that community screening is a cost-effective approach to reduce the morbidity and mortality associated with diabetes in presumably healthy individuals. On the other hand, community screening programs clearly provide a means to enhance public awareness of the seriousness of diabetes and its complications. Thus, based on expert opinion, community screening for diabetes in high-risk populations may be worthwhile, but its true efficacy is unknown. In conclusion, to increase the cost-effectiveness of testing undiagnosed, otherwise healthy individuals, screening should be considered in high-risk populations. This recommendation is consistent with the "Report of the Expert Committee on the Diagnosis and Classification of Diabetes."

Use of glycemic testing in community screening programs

If the community screening program is being implemented under the close supervision of a clinician, glycemic testing may be performed. Minors, individuals with diagnosed diabetes, and pregnant women should not receive glycemic testing in community screening programs. Pregnant women should be referred to a physician for appropriate evaluation.

Criteria for glycemic testing in community screening programs

Glycemic testing should be limited to individuals who have been found to be at high risk for having undiagnosed diabetes through the use of a verbal or written questionnaire (e.g., the Association's diabetes risk test).

Screening tests

The screening test of choice is the FPG test. Fasting is defined as no consumption of food or beverage other than water for at least 8 h before testing. An FPG level ≥126 mg/dl (7.0 mmol/l) is an indication for evaluation by a physician.

Table 2—*Criteria for the diagnosis of diabetes mellitus*

Normoglycemia	IFG or IGT	DM*
FPG <110 mg/dl	FPG ≥110 and <126 mg/dl (IFG)	FPG ≥126 mg/dl
2-h PG† <140 mg/dl	2-h PG† ≥140 and <200 mg/dl (IGT)	2-h PG† ≥200 mg/dl
		Symptoms of DM and random plasma glucose concentration ≥200 mg/dl

*A diagnosis of diabetes must be confirmed, on a subsequent day, by measurement of FPG, 2-h PG, or random plasma glucose (if symptoms are present). The FPG test is greatly preferred because of ease of administration, convenience, acceptability to patients, and lower cost. Fasting is defined as no caloric intake for at least 8 h. †This test requires the use of a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water. DM, diabetes mellitus; 2-h PG, 2-h postload glucose.

Sixteen million Americans have diabetes —and millions of them don't even know it! Take this test to see if you are at risk for having diabetes. Diabetes is more common in African Americans, Hispanics/Latinos, and American Indians. If you are a member of one of these ethnic groups, you need to pay special attention to this test.

To find out if you are at risk, write in the points next to each statement that is *true* for you. If a statement is *not true*, put a zero. Add your total score.

1. My weight is equal to or above that listed in the chart.	Yes	5	_____
2. I am under 65 years of age and I get little or no exercise during a usual day.	Yes	5	_____
3. I am between 45 and 64 years of age.	Yes	5	_____
4. I am 65 years old or older.	Yes	9	_____
5. I am a woman who has had a baby weighing more than nine pounds at birth.	Yes	1	_____
6. I have a sister or a brother with diabetes.	Yes	1	_____
7. I have a parent with diabetes.	Yes	1	_____
	TOTAL		

Scoring 3-9 points

You are probably at low risk for having diabetes now. But don't just forget about it—especially if you are Hispanic, African American, American Indian, Asian American, or Pacific Islander. You may be at higher risk in the future. New guidelines recommend everyone age 45 and over should consider being tested for the disease every three years. However, people at high risk should consider being tested at a younger age.

Scoring 10 or more points

You are at high risk for having diabetes. Only a doctor can determine if you have diabetes. See a doctor soon and find out for sure.

At-Risk Weight Chart

Height (feet/inches without shoes)	Weight (pounds without clothing)
4' 10"	129
4' 11"	133
5' 0"	138
5' 1"	143
5' 2"	147
5' 3"	152
5' 4"	157
5' 5"	162
5' 6"	167
5' 7"	172
5' 8"	177
5' 9"	182
5' 10"	188
5' 11"	193
6' 0"	199
6' 1"	204
6' 2"	210
6' 3"	216
6' 4"	221

If you weigh the same or more than the amount listed for your height, you may be at risk for diabetes. This chart is based on a measure called the Body Mass Index (BMI). The chart shows unhealthy weights for men and women age 35 or older at the listed heights. At-risk weights are lower for individuals under age 35.

Figure 1—*American Diabetes Association's diabetes risk test.*

Some screening programs use blood glucose monitoring devices (meters) approved by the Food and Drug Administration for home use. While testing of capillary blood glucose (e.g., finger-stick glucose) is not the recommended procedure, administrators of programs that use meters should practice regular quality-assurance procedures and be aware of manufacturers' recommendations regarding use of their specific testing devices. Many of these meters measure whole blood glucose and not plasma glucose. Note that plasma glucose values are 10–15% higher than whole blood glucose values.

Testing of capillary blood glucose should be done with the patient in the fasting state. Individuals with a fasting capillary whole blood glucose test result ≥ 110 mg/dl (6.1 mmol/l) should be referred to a physician for further testing. If necessary, a random test may be performed on individuals who have taken food or drink shortly before testing. An individual with a random capillary whole blood glucose test result ≥ 140 mg/dl (7.8 mmol/l) should be referred to a physician for further testing. Testing of blood with a blood glucose monitoring device intended for home use is not considered a diagnostic procedure, even if confirmed on another occasion. Thus, a screening test using whole blood glucose does not constitute the first test in the mandatory two-test procedure for diagnosing diabetes. Thus, a whole blood screening test must be confirmed two more times using plasma from a venous sample.

Table 3 defines glycemic test results that indicate additional testing and evaluation by a physician.

Other considerations for community screening programs

1. Community screening programs must follow Occupational Safety and Health Administration guidelines.
2. Personnel conducting screening programs must be adequately trained and demonstrate competency in the testing procedure used and in related program policies and procedures. The following topics should be included in a training program:

- How to screen individuals for the pres-

Table 3—*Cutoff values for screening tests that warrant additional testing and evaluation by a physician*

FPG ≥126 mg/dl (7.0 mmol/l)
or
Fasting capillary whole blood glucose ≥110 mg/dl (6.1 mmol/l)
or
Random capillary whole blood glucose result ≥140 mg/dl (7.8 mmol/l)

FPG is the preferred test. Fasting is defined as no food or drink other than water for 8 h.

ence of diabetes risk factors (e.g., administer a written or verbal questionnaire)
- Performance of the glucose test (e.g., obtaining the sample and proper use of the glucose measurement device)
- Collection, retention, and distribution of screening test data
- Infection control procedures and their rationale
- Waste disposal procedures
- Familiarity with referral procedures and community health care resources

3. Follow-up should be conducted to verify that individuals who have positive screening test results have sought medical attention. All individuals who have glycemic testing performed must be provided a written record (a paper or computerized copy retained by the organization or facility conducting the screening) containing the following information:

- Individual's name, address, and home and/or work phone numbers
- List of the individual's identified risk factors for diabetes
- The date of the test, type of test, test results, and a statement about the possibility of a false-positive or false-negative result
- For an individual with a positive screening test result, a statement that he or she should seek further medical evaluation

4. Information on alternative health care resources should be made available to individuals who do not have access to a physician.

Bibliography

American Diabetes Association: Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (Committee Report). *Diabetes Care* 22 (Suppl. 1):S5–S19, 1999

American Diabetes Association: Gestational diabetes mellitus (Position Statement). *Diabetes Care* 22 (Suppl. 1):S74–S76, 1999

Engelgau MM, Thompson TJ, Smith PJ, Herman WH, Aubert RE, Gunter EW, Wetterhall SF, Sous ES, Ali MA: Screening for diabetes mellitus in adults: the utility of random capillary blood glucose measurements. *Diabetes Care* 18:463–466, 1995

Herman WH, Smith PJ, Thompson TJ, Engelgau MM, Aubert RE: A new and simple questionnaire to identify people at increased risk for undiagnosed diabetes. *Diabetes Care* 18:382–387, 1995

Goldstein DE, Little RR, Lorenz RA, Malone JI, Nathan D, Peterson CM: Tests of glycemia in diabetes (Technical Review). *Diabetes Care* 18:896–909, 1995

National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases: *Diabetes in America*. 2nd ed. Washington, DC, U.S. Govt. Printing Office, 1995 (NIH publ. no. 95-1468)

E

Original Articles

Cervical cancer screening programs: summary of the 1982 Canadian task force report*

The Canadian Task Force on Cervical Cancer Screening Programs, which produced its first report in 1976, was reconvened by the Department of National Health and Welfare in 1980 in response to concerns expressed about the significance of new data, changing sociosexual patterns and wide variations in the implementation of the 1976 recommendations. This article is a summary of the 1982 task force report. In addition to updates of the 1976 material new sections appear on groups at risk, mathematical models of screening, quality control in screening programs, cytologic screening coverage of the Canadian population and management of patients with abnormal smears. The 1982 recommendations deal with frequency of screening, laboratory quality control and follow-up mechanisms. The task force concludes that measures to improve the quality and sensitivity of screening programs and to include women who have never been screened will be more effective in reducing mortality from carcinoma of the cervix than will attempts to increase the frequency of screening.

The task force views as unnecessary the annual screening of women over 35 years of age whose previous smears have been normal. Since younger women are sexually more active and tend to have more than one sexual partner they are at high risk. Therefore, the task force recommends annual screening for sexually active women aged 18 to 35 years.

Physicians, health care professionals and government health agencies have a role to play in informing women about the recommended intervals for cervical smears and ensuring that screening programs of adequate quality are available. Although women are primarily responsible for entering and continuing in such a program, government-sponsored registries are essential if the full potential of cervical smear programs is to be realized.

Le groupe spécial de travail sur les programmes de dépistage du cancer du col utérin, groupe qui déposa son premier rapport en 1976, a été invité par le ministère de la Santé et du Bien-être social à se réunir de nouveau en 1980 en réponse aux inquiétudes exprimées concernant la portée des données nouvellement accumulées, des changements dans les attitudes socio-sexuelles et des importantes variations dans l'application des recommandations émises en 1976. Cet article est un résumé du rapport de 1982 du groupe spécial de travail. En plus d'une mise à jour de l'information de 1976 de nouvelles sections ont été ajoutées sur les groupes à risque, les modèles mathématiques de dépistage, le contrôle de la qualité des programmes de dépistage, la population canadienne couverte par des examens cytologiques et la prise en charge des patientes ayant un frottis de dépistage anormal. Les recommandations de 1982 abordent la fréquence des examens de dépistage, le contrôle de la qualité des laboratoires et les mécanismes de surveillance. Le groupe de travail conclut que des mesures destinées à améliorer la qualité et la sensibilité des programmes de dépistage et à rejoindre les femmes qui n'ont jamais été examinées feront davantage pour réduire la mortalité due au cancer du col utérin que toute tentative pour augmenter la fréquence des examens de dépistage.

Le groupe considère inutile l'examen annuel des femmes de plus de 35 ans dont les frottis antérieurs se sont révélés normaux. Comme les femmes plus jeunes sont sexuellement plus actives et qu'elles ont tendance à avoir plus d'un partenaire sexuel, elles constituent un groupe à risque élevé. En conséquence, le groupe spécial de travail recommande l'examen de dépistage annuel des femmes sexuellement actives âgées de 18 à 35 ans.

Les médecins, les professionnels de la santé et les services gouvernement aux de santé ont leur rôle à jouer en ce qui a trait à informer les femmes des intervalles recommandés entre chaque frottis du col et à s'assurer de la disponibilité de programmes de dépistage de bonne qualité. Bien que ce soit principalement la responsabilité de la femme de joindre un tel programme et de continuer à y participer, il est essentiel que des régistres patronnés par les gouvernements soient établis si l'on veut que les possibilités des programmes d'examens cytologiques soient entièrement réalisées.

*Task force established in 1980 by the Department of National Health and Welfare. Members: Dr. R.J. Walton (chairman), consultant, Manitoba Cancer Treatment and Research Foundation, and member, honorary medical staff and former vice president, health sciences centre, Winnipeg; Dr. H.H. Allen (representative of the Society of Obstetricians and Gynaecologists of Canada), professor, department of obstetrics and gynecology, University of Western Ontario, London; Dr. G.H. Anderson (from September 1980), director of laboratories, Cancer Control Agency of British Columbia, Vancouver; Dr. D.A. Boyes (until September 1980), executive director, Cancer Control Agency of British Columbia, Vancouver; Dr. Madeleine Blanchet, president, Conseil des affaires sociales et de la famille; and former chief, service des études épidémiologiques, direction de la recherche et de la statistique, ministère des Affaires sociales, Québec; Dr. John Carmichael, professor of obstetrics and gynecology, and head, division of gynecologic oncology, Queen's University, Kingston, Ont.; Dr. D.D. Gellman (editor), vice president, medicine and education, Vancouver General Hospital; Dr. G.B. Hill, director, department of epidemiology, Provincial Cancer Hospitals Board, Edmonton; Dr. A.B. Miller, director, epidemiology unit, National Cancer Institute of Canada, University of Toronto; Dr. D.W. Thomson, professor, department of pathology, University of Toronto, and consulting pathologist, Toronto General Hospital; Eve Kassirer (coordinator), medical sociologist, Institutional and professional services division, health services directorate, health services and promotion branch, Department of National Health and Welfare, Ottawa.

Requests for reprints of the complete report and the summary to: Mrs. Eve Kassirer, Institutional and professional services division, Health services directorate, Health services and promotion branch, Department of National Health and Welfare, Ottawa, Ont. K1A 1B4

The Task Force on Cervical Cancer Screening Programs, set up in 1973 by the Conference of Deputy Ministers of Health, published its first report in 1976.[1] The task force was reconvened in 1980 by the Department of National Health and Welfare in response to the concerns of national and provincial associations, health professionals, and task force members about the significance of new data, changing sociosexual patterns and the wide variations in the implementation of the 1976 recommendations.

In 1976 the task force found evidence to support its conclusions that cervical screening, as done in Canada, had contributed to the declining mortality of carcinoma of the cervix. It recommended that health authorities support the development of cervical cancer screening programs and that all women be encouraged to participate. Further recommendations pertained to quality control and efficiency in cytology laboratories, follow-up mechanisms, including provincial registries, and uniform terminology. Perhaps the most controversial conclusion of the task force was that most women do not need annual cervical smears. The task force was criticized for recommending that women who were not at high risk and whose initial two smears, and all subsequent smears, in a properly controlled screening program were satisfactory and without significant atypia should be examined only once every 3 years to age 35 and then once every 5 years to age 60. Its recommendations concerning the size of laboratories and the establishment of registries were also criticized.[2]

In a follow-up survey in 1980 it was found that the 1976 report had stimulated an assessment of current patterns of practice internationally and across Canada;[2] this was a prime motive of the deputy ministers in convening the task force. However, the ways in which the task force's recommendations have been implemented have varied widely from province to province depending on finances, priorities and convictions.[2] Of particular concern is the fact that only Nova Scotia, Newfoundland and British Columbia have established the province-wide registries regarded by the task force as mandatory if the examination frequency is to be reduced.

Since the 1976 report new data have provided fresh insights into such questions as What sociosexual patterns of female and male behaviour precede dysplasia? Which women are at risk? How can women at risk be more precisely classified? What data exist on "high-risk" males? What proportion of preinvasive cancerous lesions regresses? Does the rate of regression vary with age? Who should be responsible for prevention, promotion of cervical smears for women at risk who seek health care for other reasons, communication of relevant sociosexual knowledge and assurance that women follow appropriate screening schedules? What assessment has there been of the proficiency of existing screening programs? What mechanisms can ensure appropriate action following the discovery of cytologic abnormalities? To what degree does the lack of national consensus on diagnostic criteria, terminology and interpretation distort reported incidence rates and affect treatment? What is the role of colposcopy? How should cost-benefit and cost-effectiveness considerations contribute to decisions on screening programs?

Mandate of reconvened task force

- To review the conclusions and recommendations of the 1976 report in the light of new data that might support or modify the conclusions on which the original recommendations were based.
- To confirm or change the original conclusions and recommendations and to arrive at new conclusions and make new recommendation if appropriate.

This article constitutes a summary of the task force's 1982 consensus report. It is based on data from national and international literature and on data from published and unpublished Canadian documents* and reflects the responses to the 1976 report by the Canadian professional community, as obtained through an informal survey† done by the task force. The complete report is available from the Department of National Health and Welfare.

In addition to updates of the 1976 material, the 1982 report includes new chapters on groups at risk, mathematical models of screening, quality control in screening programs, cytologic screening of the Canadian population and management of patients with abnormal smears. Also, the 1982 report makes recommendations on the frequency of screening, laboratory quality control and follow-up mechanisms. Above all, the task force views as unnecessary the annual screening of women over 35 years of age whose previous smears have been normal. If the frequency of examination in such women were reduced and resources deployed to concentrate on the women at risk who are not being screened, Canadian cervical cancer screening programs could become more effective.

Cervical cancer

Epidemiologic considerations

Squamous cell carcinoma of the cervix almost always occurs in women who have been sexually active.[1] The most important risk factors are generally regarded as being early age at the time of first intercourse and multiple sexual partners.[1] Recently the role of the high-risk male in the transmission of an etiologic agent to his partner has been emphasized.[3]

Epidemiologic evidence now suggests that although infection with herpes simplex virus type 2 is not essential for cancer of the cervix, it may lead to a premalignant change in the cervical epithelial cells in a large majority of cases.[3] In addition, recent studies suggest that cigarette smoking may play an etiologic role.[4]

Natural history

The 1976 report suggested a somewhat longer natural

*Mortality data from Statistics Canada (*Causes of Death*, cat no 84-203) and unpublished tabulations of the British Columbia Central Cytology Registry; incidence data from Statistics Canada (*Cancer in Canada*, cat no 82-207) and the British Columbia Central Cytology Registry; screening coverage data from the Canadian Society of Cytology (unpublished data from a 1980 survey); and data relative to quality control in cytologic screening programs from the Ontario Laboratory Proficiency Testing Program (collected from 1977 to 1981).

†Of provincial medical associations, cancer foundations and institutes, and university departments of pathology, obstetrics and gynecology, and family and community medicine.

history of carcinoma in situ than had been suspected previously, an interpretation based on an assumption of the progressive nature of the disease.[1] New data from a British Columbia cohort study indicate that sometimes carcinoma in situ, especially in younger women, regresses.[5] In addition, anecdotal evidence suggests that today a greater proportion of cases may be rapidly progressive, again especially in younger women.[6]

Efficacy of screening programs

The reconvened task force has found that the rate of fall in the incidence of cervical cancer in all provinces that was reported in 1976 has slowed, at least in women under the age of 65. Were is not for the screening programs in Canada the registered incidence of invasive cancer might actually have risen. Further, a case–control study of patients in Ontario supports the effectiveness of the screening programs.[7]

Perhaps the most convincing evidence is the comparison of the trends in incidence in Norway with those in Denmark, Sweden, Finland and Iceland.[8] Only in Norway, where a screening program has not been introduced, has there been no decrease in incidence.

In accord with the report by Miller and colleagues[9] the original task force concluded that cytologic screening programs were becoming effective in reducing the mortality of carcinoma of the cervix; this reduction was directly related to the proportion of the population screened.[9] At that time mortality data were available only up to 1972. When the analysis was extended to 1974–76 the correlation between changes in screening index and reductions in mortality disappeared.[10] It may be that once the screening index reaches a certain level

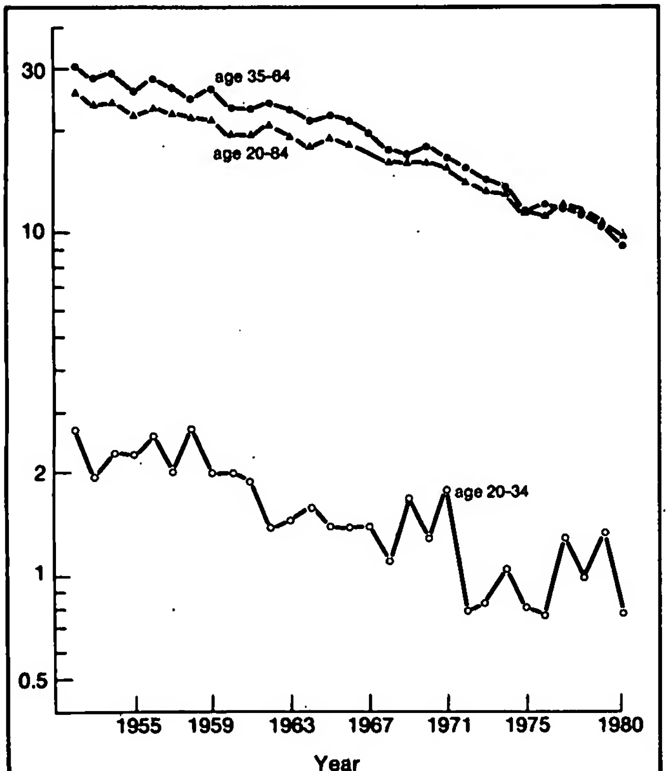


FIG. 1 — Rates of death from cancer of the uterus, age-standardized per 100 000 women, Canada 1952–80.

further increases lead only to a marginal reduction in mortality, reflecting repeat examinations of the same women rather than examinations of previously unscreened women, who are at highest risk.

The trends in mortality for women aged 20 to 34, 35 to 64 and 20 to 84 years (standardized for age but uncorrected for hysterectomies) in Canada from 1952 to 1980 are shown in Fig. 1. Data for "malignant neoplasm of cervix uteri" and "other malignant neoplasms of the uterus" were combined because of the decreasing proportions coded as "malignant neoplasm of the uterus, unspecified". Other data suggest that the contribution of cancer of the endometrium to the total rate of death from uterine cancer in women under the age of 65 years is very small and almost stable. It appears from Fig. 1 that the decrease in mortality is continuing but may have slowed.

Table I shows that the rates of death from cancer of the uterus (cancer of the cervix uteri, corpus uteri and uterus, unspecified, combined[11]) of women aged 35 to 64 years in each province for 1957–59, 1967–69 and 1977–79 are still declining, with a continued east–west differential. Therefore, the task force concludes that squamous cell carcinoma of the cervix can be controlled by means of a cytologic screening program for the following reasons:

- Invasive squamous cell carcinoma of the cervix is preceded by a spectrum of disease, extending over many years, that may be recognized at the stages of dysplasia and carcinoma in situ.
- In a significant proportion of patients with severe dysplasia or carcinoma in situ the disease, if untreated, will develop into invasive squamous cell carcinoma.
- Cytologic evidence of dysplasia and carcinoma in situ can be easily, safely and economically obtained by the preparation and examination of smears.
- Once dysplasia or carcinoma in situ has been identified further progress of the disease can be prevented by simple therapeutic procedures and continuing surveillance.

Groups at risk

The reconvened task force reinforces the view that, on the basis of epidemiologic and sociologic evidence, women can be classified into two groups according to their risk of cervical cancer. The group not at risk,

Table I—Rates of death from cancer of the uterus,* according to province

Province	Mortality† 1957–59	1967–69	1977–79
British Columbia	21.6	14.7	11.3
Alberta	20.2	11.8	9.4
Saskatchewan	12.3	12.0	7.9
Manitoba	21.5	11.6	9.3
Ontario	23.7	17.2	11.4
Quebec	33.0	21.3	11.0
New Brunswick	25.2	21.8	11.6
Nova Scotia	32.8	25.8	16.3
Prince Edward Island	28.8	24.3	19.7
Newfoundland	36.4	23.5	18.4

*Includes cancer of the cervix uteri (ICD 180), corpus uteri (ICD 182.0) and uterus, unspecified (ICD 182.9).[11]
†Age-standardized rates of death per 100 000 women aged 35 to 64 years.

which should not be included in a screening program, includes women who have never had sexual intercourse, those over 60 years of age for whom previous smears have always been negative, and those who have had a hysterectomy for benign disease (with adequate documentation that all of the cervical epithelium was removed). The group at risk includes all other women.

The 1976 task force report identified a subgroup of women at high risk: those who had had intercourse at an early age and those who had had multiple sexual partners.[1] The 1982 task force report considers that this subgroup probably also includes women whose husbands or other regular sexual partners have had multiple partners. Because it has become increasingly common for women and men to have more than one sexual partner, and because women up to age 35 are the most active sexually, many women born after 1945 will probably fall into the high-risk subgroup.

There is as yet no evidence that the use of oral contraceptives alone increases the risk of cancer of the cervix.

It cannot be emphasized too strongly that women who are found to have dysplasia or carcinoma in situ must be removed from the screening program and managed according to the severity of the lesion.

The reconvened task force believes that information on the degree of risk of cancer of the cervix should be generally available — for example, in physicians' offices. Special attempts should continue to be made to screen women who are attending clinics for individuals with sexually transmitted diseases or who are admitted to penal institutions.

Cytologic screening programs

Mathematical models

Mathematical models of cancer screening can be used in three ways: to numerically describe the impact of a particular disease in a defined population (modelling the natural history); to quantify the effect of screening on the natural history (detection of the disease or its precursors in asymptomatic individuals); and to determine optimal screening schedules, with a criterion of desired effect (e.g., reduction in mortality or increase in numbers of potential years of life saved).

It is difficult to summarize the results of the various models of cancer screening[12-16] since assumptions made and the criteria used to determine optimal effect differ so much. However, it is possible to make some general conclusions.

First, the models confirm what is intuitively obvious — namely, that there are diminishing returns with increasing frequency of screening in a given cohort of women. Second, in light of the diminishing returns, and from the point of view of public health, it is better to spend money on increasing the number of women being screened for the first time than on increasing the frequency of screening for women who have already been screened. Third, and again from the point of view of public health, establishing a policy in which screening is done on the basis of the current screening history of the target population is more beneficial than prescribing a lifetime schedule for all women. Obviously, this advantage can be gained only if screening programs are organized on a community basis. Fourth, improving the quality and sensitivity of screening programs will be more effective than increasing the frequency of screening in reducing mortality.

Screening schedules

In both the 1976 and the 1982 reports the task force, in discussing screening schedules, considered onset of screening, frequency of screening and age when screening is stopped. Internationally, there are many variations in screening schedules.[17-20] For the 1982 report the task force re-examined optimum schedules largely from an assessment of mathematical models, particularly through the application of influences on natural history, derived from the British Columbia cohort study,[5] and recent Canadian trends in incidence and mortality, according to the model of Knox.[12] Full details of the simulations are given by Yu and associates,[21] and some of their results are shown in Table II.

Table II—Summary of results of computer simulation of screening programs*

Variable	Screening schedule: Age at onset (yr)	Interval between screens (yr)†	Age when stopped (yr)	Total no. of tests per lifetime	No. of deaths per 100 000 women	Percentage reduction in mortality
Women at risk						
				0	108.3	
	28	3 and 5‡	55	8	9.4	91
	25	3 and 5‡	60	10	7.1	93
	22	3 and 5‡	65	12	6.4	94
	20	3 and 5§	60	11	7.8	93
	25	3	59	12	5.4	95
	25	1 and 2¶	61	22	1.7	98
Women at high risk						
				0	132.7	
	25	3 and 5‡	60	10	28.6	78
	20	3 and 5§	60	11	11.0	92
	20	3	53	12	9.2	93
	25	1 and 5§	60	16	3.1	98
	20	1 and 5§	60	21	1.3	99
	20	1 and 5§	60	24	1.1	99

*Based on findings of Yu and associates.[21]
†Frequency changed at age 40,‡ 35§ or 31¶ years.

Given the difficulties of identifying and classifying women at high risk, the probability that many young women are now at high risk, the natural history of the disease and the need to provide consistent recommendations for all age groups, the reconvened task force interprets the results of mathematical modelling as reinforcing the general validity of the 1976 recommendations. In general, Canadian women aged 35 years or more have now entered screening programs and should require screening no more than once every 5 years. Since younger women are more sexually active and tend to have more than one partner they are at high risk; therefore, annual screening is justifiable until age 35. The specific changes proposed to the 1976 recommendations are therefore:

- No attempt should be made to categorize women at high risk on a group basis.
- Women who have had sexual intercourse should generally be advised to be screened annually between 18 and 35 years of age.
- Women over the age of 35 years who are considered at high risk, by their contact with venereal disease clinics or penal institutions or by their own judgement or that of their physicians, should not be discouraged from having smears more than once every 5 years if they request them.

However, the recommendation of the 1976 report — that women over the age of 35 years should, in general, be advised that they should be screened every 5 years — still holds.

It should again be emphasized that these recommendations on screening frequency over the age of 35 are based on the availability of an appropriate registry mechanism by which surveillance can be maintained to ensure that the recommendations on frequency are not translated into less frequent screening by default. Further, they require that physicians or others who know how to take adequate smears be available and that skilled cytology assessment be done in laboratories with adequate quality control programs. The recommendations apply only to women whose smears are repeatedly negative for epithelial atypia. Whenever changes are detected on a cervical smear repeat examinations should be dictated by the requirements for surveillance, diagnosis, treatment and adequate follow-up of the abnormalities identified.

Table III—Extent of cervical screening performed in Canada in 1971, 1975, 1977 and 1979

Province	No. of screens per 100 women aged 20 years or more*			
	1971	1975	1977	1979
British Columbia	55	61	55	57
Alberta	40	48	47	48
Saskatchewan	41	47	48	47
Manitoba	46	51	50	48
Ontario	39	45	44	44
Quebec	29	42	43	45
New Brunswick	22	41	37	40
Nova Scotia	37	35	45	46
Prince Edward Island	28	37	41	44
Newfoundland	36	43	42	44
Canada	38	45	45	46

*Not to be equated with the number of women screened.

The malignant diseases that most frequently affect women in Canada — cancer of the breast, large bowel, ovary, lung and endometrium — occur in women who are 20 to 30 years older than those to whom screening programs for carcinoma of the cervix and its precursors should be directed. Thus, although opportunities created by screening or medical examinations for these diseases should be used for taking cervical smears that are due to be taken, the frequency of cervical smears should not be affected by these opportunities.

Measures to improve the quality and sensitivity of screening programs and to include women who have never been screened will be more effective than attempts to increase the frequency of screening in reducing the mortality of carcinoma of the cervix.

A cytologic screening program will be most effective if:

- Smears are obtained by medical or specially trained paramedical personnel.
- Smears are correctly identified and accompanied by the information required by the program.
- Smears are screened for abnormalities by qualified and experienced cytotechnologists under the supervision of cytopathologists.
- Suitable quality control is exercised in the laboratory.
- Precise terminology is used.
- Results of the examinations are reported to the patient's physician or other person responsible for the patient's health care.
- Follow-up mechanisms exist to ensure appropriate action.

Screening of the Canadian population

Table III shows the cytologic screening coverage in Canada of women aged 20 years and over for 1971, 1975, 1977 and 1979; only data for 1971 were included in the 1976 report. The figures are based on the number of cytologic examinations (not the number of women screened) in each province, as determined in a recent survey by the Canadian Society of Cytology (unpublished data, 1980). There was a significant increase in Quebec and the Atlantic provinces in the number of examinations performed up to the mid-70s. By 1979 the proportions were remarkably uniform across Canada, although the proportion in British Columbia was still higher.

Quality control

Although little has been done in Canada to develop quality control programs for cytologic testing, the Laboratory Proficiency Testing Program in Ontario has accumulated data from 1977 to 1981 on the diagnostic accuracy of testing of all cytology laboratories (Table IV). Further analysis of the data, not displayed here, indicates that proficiency varies considerably between laboratories handling similar annual numbers of cases. The findings have been made available to the laboratories, and appropriate educational programs have been started.

There are several links in the chain of cytologic screening:

Avoidance of false-negative reports on cervicovaginal smears: Detailed information is provided in the full 1982 task force report under the following categories of causes of such report:

- Error in documentation of smear.
- Inadequate or improper sampling of a lesion.
- Presence of interfering substances.
- Technical problems in processing.
- Errors in screening and reporting.

Adequate cervicovaginal smears: Over the past 20 years there has been much controversy concerning the most satisfactory technique for sampling the cervix and the criteria by which a cytologic sample should be categorized as adequate or inadequate. Within the spectrum of cervical dysplasias and cancers the lesions differ in such features as cell type, location, area of onset of invasion, modification by keratinization, occurrence either within or separate from condylomatous lesions, overall extent and tendency to be focal or multifocal. In view of these variables and the fact that much of the yield of screening in recent years has been cases of mild or moderate dysplasia, in which the abnormality is usually small or localized, it is important that the direct cervical sample be circumferential. It should be adjusted when necessary to accommodate any grossly evident variation in the location of the original squamous columnar junction or in the extent of the transformation zone.

Proposed reporting terminology: Recognizing the need for more uniform terminology in reporting the results of cervical cytologic examinations the 1976 report recommended a standard nomenclature, which is reproduced in the 1982 report with two important additions: "atypical metaplasia" and "condyloma effect" have been added to the category "abnormal cells consistent with benign atypia (nondysplastic)" as a result of the advances in our understanding of cytologic abnormalities over the past few years. Further, the terms recommended for "abnormal cells consistent with dysplasia" are "mild dysplasia", "moderate dysplasia" and "severe dysplasia". The task force prefers this terminology to the alternative CIN (cervical intraepithelial neoplasia) terminology.

Population registries

The 1976 report recommended that provincial registries be established to record data and institute the following recommendations:

That normal patients are recalled at regular intervals for repeat smears according to the guidelines of the program.

That long-term follow-up be provided for patients who have received treatment following the diagnosis of an abnormality.

Such a system can only be effected through a centralized registry. In Canada, provincial registries should establish uniform data-processing systems so that interprovincial communication and comparisons will be possible.

Perhaps those who considered the 1976 recommendations failed to recognize that they were an integral and complete package and that the recommendations on frequency should not be considered in isolation. Only in Nova Scotia has a provincial registry been established since the 1976 report, although registries had been established in Newfoundland and British Columbia before 1976. In Quebec two large laboratories (one in Montreal and one in Quebec City) have established registries, but they are not large enough to include the total population of the province and do not have the resources to carry out all the other recommendations. A provincial cytology register in Manitoba, which was not carrying out the functions intended by the task force, has even been abolished since 1976.[2]

As with all preventive medicine programs it is necessary to consider who is responsible for ensuring that women enter cervical screening programs and have cervical smears taken at the recommended intervals. The reconvened task force believes it is primarily the responsibility of the women. However, physicians, other health care professionals and government public health agencies have a role to play in educating women.

Table IV—Data from Ontario Laboratory Proficiency Testing Program for gynecologic cytology, 1977–81*

	No. of laboratory diagnoses reported on test slides									% of laboratory diagnoses that deviated significantly†		
	Degree of deviation from committee diagnosis											
	Undercall				Overcall							
Committee diagnosis	≥ 3	2	1	0	1	2	≥ 3	None	Total	Undercall	Overcall	Total
No abnormal cells	NA	NA	34	511	112	12	4	4	677	NA	2.4	2.4
Benign atypia	NA	NA	173	913	96	11	4	5	1202	NA	1.2	1.2
Mild dysplasia	NA	39	119	133	27	1	1	2	322	12.1	0.6	12.7
Moderate dysplasia	8	58	96	102	48	26	NA	1	339	19.5	7.7	27.2
Severe dysplasia	31	27	57	54	52	5	NA	0	226	25.7	2.2	27.9
Malignant squamous cells	56	35	91	300	14‡	NA	NA	0	496	18.3	NA	18.3

*Testing was carried out by circulation of chosen test slides to all 160 laboratories processing gynecologic cytology specimens. These data are a cumulation from the first four complete surveys. NA = not applicable.

†Were at least two degrees different from the committee diagnosis.

‡An additional type of malignant abnormality was recorded by the laboratory but not categorized as such by the committee (e.g., sarcoma cells in addition to malignant squamous cells).

Primary care physicians could set up reminder mechanisms for their female patients who fail to return for a smear. It has been suggested that the licensing regulations in some provinces are interpreted to mean that physicians are not permitted to invite women to return for repeat smears if previous smears were negative. The task force therefore recommends that provincial licensing bodies should ensure that their medical acts and regulations are amended, if necessary, to permit physicians to remind their patients that the time has come for them to have a repeat cervical cytology examination — or, indeed, any other recognized preventive medical procedure.

It has also been suggested that provincial registries with a follow-up function would be expensive and difficult to run and might be regarded as an invasion of privacy. The task force rejects such arguments. Modern computer technology enables them to be run economically; in fact, such registries have already been used elsewhere to ensure adequate compliance with vaccination schedules in children.[22]

Given the fallibility of the human memory and the mobility of the Canadian population, the task force believes that government-sponsored registries are essential if the full potential of cervical screening programs is to be realized. It is not for the task force to determine how a cytology registry might be established in any province. This will depend on existing resources and personnel. However, the registries could be linked with provincial or regional cytology laboratories, provincial cancer registries or existing record systems of provincial medical insurance schemes. Once again, these recommendations should not be lost by default.

Relation to other screening programs

Several critics of the 1976 recommendations felt that whatever the merits of an annual cervical examination, women should be encouraged to see their physicians at least once a year to be examined for other conditions. However, the task force concluded that the frequency of cervical cytologic screening should be determined on its own merits and not with a view to conducting an annual examination for other purposes. On the other hand, when more than one examination is indicated it should be coordinated with the other and with other necessary visits to the physician's office or public health clinic.

Management of patients with abnormal cervical smears

The 1976 report mentioned the developing role of colposcopy in assessing patients with abnormal cervical smears. Since then its role has become more clearly defined. While cytologic screening detects preclinical cervical dysplasia or neoplasia, colposcopic examination evaluates the cervix with abnormal cells and enables the colposcopist to select the site for biopsy, which usually permits a determination of whether invasive cervical cancer is present.[23] Colposcopy is particularly valuable for assessing cervical abnormalities in young women. With an adequate biopsy it avoids not only the inherent morbidity of cone biopsy and the hospital stay required, but also the potential adverse effects of cone biopsies on reproductive function.[24,25] Such biopsies can now be limited to patients in whom colposcopic examination is unsatisfactory, those in whom colposcopically directed biopsies, performed to rule out more advanced disease, have identified "microinvasion", and those who continue to show significant abnormal findings, the source of which cannot be identified colposcopically.[23,26,27]

Women whose cervical smears suggest moderate to marked dysplasia, carcinoma in situ or invasive disease should undergo colposcopic examination. Women whose smears show mild dysplasia should have the smear repeated every 2 or 3 months, and if the dysplasia persists they should undergo colposcopic examination since significant dysplasia or carcinoma in situ is not uncommonly found in specimens obtained by colposcopically directed biopsy in such patients.

There is disagreement as to what constitutes proper management of patients with cervical dysplasia or carcinoma in situ. In general, when mild or moderate dysplasia is found by colposcopic examination and the limits of the lesions have been identified the patient may be treated by conservative measures such as electrocauterization or cryotherapy. Severe dysplasia and carcinoma in situ may be managed conservatively in selected patients,[28,29] or by therapeutic conization or hysterectomy.[30] Treatment will depend on the size and location of the lesion, the patient's age, parity and reliability for follow-up, and other medical and gynecologic considerations.

When microinvasive carcinoma is diagnosed colposcopically a diagnostic cone biopsy should be done to rule out more advanced disease. There is no standard pathologic definition as to what constitutes microinvasive carcinoma (stage Ia). However, a simple hysterectomy is generally considered an adequate form of treatment.[31,32] Occult preclinical invasive carcinoma (stage Ib) is treated either by irradiation or by radical hysterectomy and pelvic lymphadenectomy, depending on the clinical findings, the patient's age and the treatment policy of the centre.

It is clear that colposcopy offers a degree of flexibility in the evaluation and management of patients with preclinical cervical neoplasia that is not available with diagnostic conization. It is crucial, however, that early invasive carcinoma of the cervix not be missed by colposcopic assessment, since this would lead to inappropriate conservative therapy.[33,34] Colposcopy imposes a great responsibility on the colposcopist to accurately evaluate the cervix with abnormal cells.

Recommendations

The recommendations* of the task force should be taken as a whole. It is inappropriate to establish the recommended screening frequencies as formal policy in the absence of established systems to monitor the frequencies and to issue reminders to attend at the recommended intervals in the absence of proper quality control systems.

On the basis of its conclusions the task force recommends the following:

I. Health authorities should encourage and support the development of cytologic screening programs de-

*Some of the following recommendations are unchanged from the 1976 report; therefore, readers are referred to that report for the rationales.

signed to detect the precursors of clinical invasive carcinoma of the cervix.

II. Women who have had sexual intercourse should generally be advised to have screening annually between the ages of 18 and 35 years and thereafter every 5 years until age 60.

III. Women over the age of 60 who have had repeated satisfactory smears without significant atypia may be dropped from a screening program for squamous cell carcinoma of the cervix.

IV. High priority should be given to encouraging women at risk who have never had a cervical cytology examination to have one and to enter a screening program.

V. The recommended screening frequencies apply only to women whose smears show no epithelial cell atypia. Once such changes are detected schedules for repeat examinations should be dictated by the requirements for surveillance, diagnosis, treatment and follow-up.

VI. Appropriate means should be employed to inform women of their degree of risk of carcinoma of the cervix. However, no attempt should be made to categorize women at high risk on a group basis.

VII. Women over 35 years of age whose contact with the health care system is through venereal disease clinics or penal institutions and who, in their own judgement or that of their physicians, are at high risk should not be discouraged from having smears more frequently than every 5 years if they request them.

VIII. Provincial mechanisms that establish young women's independent responsibility for health insurance purposes (usually at or about the age of 19 years) should be used to remind women that if they are sexually active they should have a cervical smear if they have not already done so.

IX. Individual screening examinations for various diseases should be promoted on their own merits and performed at the most cost-effective frequency. When two or more screening examinations are being offered, each on its own merits, to the same population group cost-effectiveness considerations may require a change in timing for some of them.

X. Colposcopic examination should not be used as a screening method: it is an important diagnostic tool for localizing and assessing premalignant disease and early invasive carcinoma of the cervix in women with abnormal cervical smears. Colposcopy should be performed only by gynecologists with adequate training and experience in the technique. They should work in a clinical setting that provides a continuing source of abnormal material so that their expertise can be maintained and enhanced.

XI. To function most efficiently within a mass screening program a laboratory should process a sufficient number of cases* annually to require staffing by a minimum of three qualified and experienced cytotechnologists, supervised by a cytopathologist, and adequate clerical and technical support staff.

*Although in the 1976 report 25 000 smears was considered a "sufficient number" (on the basis of efficiency, not proficiency), this number is deleted in the 1982 report. Disagreement with the deletion was registered by one task force member, Dr. A.B. Miller.

XII. The jurisdictions responsible for such screening programs should ensure that appropriate mechanisms for quality control have been established and are functioning satisfactorily.

XIII. Uniform terminology, such as that recommended in this report, should be used for reporting smears and biopsies and for recording data in cancer registries so as to assist in the exchange of information from one laboratory to another and to ensure that the various provincial registries are provided with data that are reported in a uniform manner.

XIV. All mass screening programs should have follow-up systems to ensure that:

(a) People with normal test results are recalled at regular intervals for repeat testing according to the guidelines of the program.

(b) Action is taken following the discovery of an abnormality.

(c) Long-term follow-up is provided for patients who have received treatment following the diagnosis of an abnormality.

Such a system can only be effected through a central registry. Provincial registries should establish compatible data-processing systems so that interprovincial communication and comparisons will be possible.

XV. Provincial licensing bodies should ensure that provincial medical acts and regulations permit physicians to remind their patients when it is time for repeat cervical cytology examinations — or, indeed, any other recognized preventive medical procedure.

XVI. Departments and ministers of health should consult with the appropriate bodies in their jurisdictions with a view to selecting the best mechanisms to establish functional registries of all cervical cytology examinations as soon as possible.

XVII. The deputy minister of national health and welfare should make available the resources of the Department of National Health and Welfare to coordinate the establishment of provincial cervical cytology registries and thus facilitate the exchange of data and the transfer of records when women move from one province or region to another.

We thank Dr. Eve Shulman, epidemiologist, health assessment division, health services and promotion branch, Department of National Health and Welfare for her resource assistance.

References

1. Task force convened by the Department of National Health and Welfare: Cervical cancer screening programs. *Can Med Assoc J* 1976; 114: 1003–1033

2. KASSIRER E: Impact of the Walton report on cervical cancer screening programs in Canada. *Can Med Assoc J* 1980; 122: 417–423

3. MILLER AB, RAWLS WE: The epidemiology of gynecologic cancer: I. cervix. In COPPLESON M (ed): *Gynecologic Oncology: Fundamental Principles and Clinical Practice*, Churchill, London, 1981: 9–18

4. CLARKE EA, MORGAN RW, NEWMAN AM: Smoking as a risk factor in cancer of the cervix: additional evidence from a case-control study. *Am J Epidemiol* 1982; 115: 59–66

5. BOYES DA, MORRISON B, KNOX EG, DRAPER GJ, MILLER AB: A cohort study of cervical cancer screening in British Columbia. *Clin Invest Med* 1982; 5: 1–29

6. BERKELEY AS, LIVOLSI VA, SCHWARTZ PE: Advanced squamous cell carcinoma of the cervix with recent normal Papanicolaou tests. *Lancet* 1980; 2: 375–376

7. CLARKE EA, ANDERSON TW: Does screening by "Pap" smears help prevent cervical cancer? A case-control study *Lancet* 1979; 2: 1–4

8. HAKAMA M: Trends in the incidence of cervical cancer in the Nordic countries. In MAGNUS K (ed): *Trends in Cancer Incidence*, Hemisphere Pub, Washington, 1982: 279–292

9. MILLER AB, LINDSAY J, HILL GB: Mortality from cancer of the uterus in Canada and its relationship to screening for cancer of the cervix. *Int J Cancer* 1976; 17: 602–612

10. MILLER AB, VISENTIN T, HOWE GR: The effect of hysterectomies and screening on mortality from cancer of the uterus in Canada. *Int J Cancer* 1981; 27: 651–657

11. *International Classification of Diseases*, 8th rev, WHO, Geneva, 1969

12. KNOX EG: A simulation system for screening procedures. Computer simulations of cervical cytology screening programmes. In *The Future and Present Indicatives. Problems and Progress in Medical Care*, 9th ser, Nuffield Provincial Hospitals Trust, Oxford, 1973: 19–55

13. GALLIHER HP: *Cost-effective Planned Lifetime Schedules of Pap Smears: Estimated Maximal Potentials*, Michigan Cancer Foundation, Detroit, 1976

14. EDDY DM: *Rationale for the Cancer Screening Benefit Program. Screening Policies. Implementation Plan: Part III*, Blue Cross Assoc, Chicago, 1977

15. SCHWEITZER SO, LUCE BR: *A Cost-effective Approach to Cervical Cancer Detection*, US Dept of Health and Human Services, Public Health Service, Office of Health Research, Statistics and Technology, National Center for Health Services Research, DHEW publ no (PHS) 79-3237, Hyattsville, Md, 1979

16. ALBERT AL, GERTMAN PM, LOUIS TA, LIU SI: *Screening for the Early Detection of Cancer II. The Impact of Screening on the Natural History of the Disease*, dept of mathematics, Boston U, Boston, 1977

17. SPRIGGS AI, HUSAIN OA: Cervical smears. *Br Med J* 1977; 1: 1516–1518

18. Guidelines for the case-related check-up: recommendations and rationale. *CA* 1980; 30: 194–240

19. HAKAMA M: Mass screening for cervical cancer in Finland. In MILLER AB (ed): *Screening in Cancer, A Report of a UICC International Workshop, Toronto, Canada, April 24–27, 1978* (UICC tech rep ser, vol 40), International Union Against Cancer, Geneva, 1978: 93–107

20. JOHANNESSON G, GEIRRSON G, DAY N: The effect of mass screening in Iceland, 1965-74, on the incidence and mortality from cervical carcinoma. *Int J Cancer* 1978; 21: 418–425

21. YU S, MILLER AB, SHERMAN GJ: Optimising the age, number of tests and test interval for cervical screening in Canada. *J Epidemiol Community Health* 1982; 36: 1–10

22. BUSSEY AL, HARRIS AS: Computers and the effectiveness of the measles vaccination campaign in England and Wales. *Community Med* 1979; 1: 29–35

23. STAFL A, MATTINGLY RF: Colposcopic diagnosis of cervical neoplasia. *Obstet Gynecol* 1973; 41: 168–176

24. HESTLER LL JR, READ RA: An evaluation of cervical conization. *Am J Obstet Gynecol* 1960; 80: 715–721

25. STAFL A, MATTINGLY RF: Cervical intraepithelial neoplasia. In MATTINGLY RF (ed): *Te Lindes' Operative Gynaecology*, 5th ed, Lippincott, Philadelphia, 1977: 671–691

26. TOWNSEND DE, OSTERGARD DR, MISHEL DR JR, HIROSE FM: Abnormal Papanicolaou smears. Evaluation by colposcopy, biopsies and endocervical curettage. *Am J Obstet Gynecol* 1970; 108: 429–434

27. TREDWAY DR, TOWNSEND DE, HOVLAND DN, UPTON RT: Colposcopy and cryosurgery in cervical intraepithelial neoplasia. *Am J Obstet Gynecol* 1972; 114: 1020–1024

28. KAUFMAN RH, CONNER JS: Cryosurgical treatment of cervical dysplasia. *Am J Obstet Gynecol* 1971; 109: 1167–1174

29. ODELL LD: Electrocautery for the treatment of early cervical neoplasia. *J Reprod Med* 1973; 10: 81–84

30. KOLSTAD P, KLEM V: Long-term followup of 1121 cases of carcinoma in situ. *Obstet Gynecol* 1976; 48: 125–129

31. AVERETTE HE, NELSON JH JR, NG AB, HOSKINS WJ, BOYCE JG, FORD JH: Diagnosis and management of microinvasive (stage IA) carcinoma of the uterine cervix. *Cancer* (suppl) 1976; 38: 414–425

32. KOLSTAD P: Carcinoma of the cervix stage IA — diagnosis and treatment. *Am J Obstet Gynecol* 1969; 104: 1015–1022

33. KOHAN S, BECKMAN EM, BIGELOW B, CARP M, DOUGLAS GW: Colposcopy in the management of cervical intraepithelial neoplasia (E). *Obstet Gynecol Surv* 1977; 32: 621–623

34. SEVIN BU, FORD JH, GIRTANNER RD, HOSKINS WJ, NG AB, NORDQVIST SR, AVERETTE HE: Invasive cancer of the cervix after cryosurgery. Pitfalls of conservative management. *Obstet Gynecol* 1979; 53: 465–471

Available online at www.sciencedirect.com
SCIENCE DIRECT

GENOMICS

Genomics 87 (2006) 693–703

www.elsevier.com/locate/ygeno

F

Genomic profiles for human peripheral blood T cells, B cells, natural killer cells, monocytes, and polymorphonuclear cells: Comparisons to ischemic stroke, migraine, and Tourette syndrome

XinLi Du [a,b], Yang Tang [a,b], Huichun Xu [a,b], Lisa Lit [a,b], Wynn Walker [a,b], Paul Ashwood [a,d], Jeffrey P. Gregg [a,c], Frank R. Sharp [a,b,*]

[a] *MIND Institute, University of California at Davis, 2805 50th Street, Sacramento, CA 95817, USA*
[b] *Department of Neurology, University of California at Davis, 2805 50th Street, Sacramento, CA 95817, USA*
[c] *Department of Pathology, University of California at Davis, 2805 50th Street, Sacramento, CA 95817, USA*
[d] *Department of Medicine, University of California at Davis, 2805 50th Street, Sacramento, CA 95817, USA*

Received 3 November 2005; accepted 5 February 2006
Available online 20 March 2006

Abstract

Blood genomic profiling has been applied to disorders of the blood and various organ systems including brain to elucidate disease mechanisms and identify surrogate disease markers. Since most studies have not examined specific cell types, we performed a preliminary genomic survey of major blood cell types from normal individuals using microarrays. $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, $CD56^+$ natural killer cells, and $CD14^+$ monocytes were negatively selected using the RosetteSep antibody cocktail, while polymorphonuclear leukocytes were separated with density gradient media. Genes differentially expressed by each cell type were identified. To demonstrate the potential use of such cell subtype-specific genomic expression data, a number of the major genes previously reported to be regulated in ischemic stroke, migraine, and Tourette syndrome are shown to be associated with distinct cell populations in blood. These specific gene expression, cell-type-related profiles will need to be confirmed in larger data sets and could be used to study these and many other neurological diseases.

Keywords: Blood; Humans; Gene expression; Microarrays; Genome; T cells; B cells; NK cells; Neutrophils; Migraine; Stroke; Tourette

Gene expression profiling of peripheral blood using microarrays has been applied to malignant and immune disorders, including leukemia, lymphoma, systemic lupus erythematosis, rheumatoid arthritis, and many others [1–4]. This approach has helped identify important diagnostic and prognostic markers as well as potential therapeutic targets. This approach has also been extended to many diseases of other organ systems. It is likely that many inflammatory, autoimmune, and genetic factors could affect gene expression of peripheral blood cells without causing overt changes to hematological and immunological phenotypes. Proof-of-principle blood genomic studies have been performed in animals [5] and humans [6,7]. Subsequent studies have demonstrated characteristic blood genomic patterns for acute ischemic stroke [8], migraine headache [9], Tourette syndrome [10], renal cell carcinoma [11], multiple sclerosis [12], benzene exposure [13], trauma [14], and neurogenetic disorders including neurofibromatosis type I, tuberous sclerosis type II, Down syndrome [7,15], and Huntington chorea [16]. The study of blood gene expression profiles appears to be a promising approach that may provide mechanistic insights and surrogate markers for many diseases.

Several blood RNA isolation methods have been used to date. These include methods starting with whole blood, mononuclear cells, and buffy coat [6,7,11,14,17,18]. However, the RNA isolated using these methods comes from various blood cell subsets that originate from different

* Corresponding author. MIND Institute, University of California at Davis, 2805 50th Street, Sacramento, CA 95817, USA. Fax: +1 916 703 0369.
E-mail address: frank.sharp@ucdmc.ucdavis.edu (F.R. Sharp).

0888-7543/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygeno.2006.02.003

developmental lineages, perform separate and distinct biological functions, and, most likely, have very different genomic expression signatures. It has been recognized that age and gender and the different composition of blood cells from each individual represent a major source of normal variation of blood gene expression [6,7]. In addition, a disease may predominantly affect one specific blood cell subtype while sparing others. Therefore, characterizing the contribution of every blood cell subtype to the overall blood genomic pattern may be essential to distinguish significant genomic changes from noise, interpret the disease-related patterns, and decide on the proper blood cell types to perform follow-up confirmatory analyses.

Expression profiles of blood cells such as T lymphocytes [14,19] and platelets [18,20] have been described. However, studies that compare directly the whole genomic expression profiles of several major blood cell subtypes have not been performed in detail. In this study, we attempted to build a preliminary gene expression database by comparing major leukocyte subsets from three healthy donors, including polymorphonuclear cells (PMN), monocytes, B cells, $CD4^+$ T cells, $CD8^+$ cytotoxic T cells, and natural killer (NK) cells to determine whether there is likely to be a unique expression signature of each cell type. To demonstrate the utility of these expression signatures, we applied these data to the whole blood genomic profiles of several neurological diseases that we have studied previously, including acute ischemic stroke [8], migraine [9], and Tourette syndrome [10], to demonstrate that the blood genomic signatures of each of these conditions can be ascribed to certain blood cell subtypes being affected by each disease. Future studies likely could determine not only whether hematological and systemic diseases affect gene expression in specific subsets of blood cells, but also whether the diseases affect specific signaling pathways in specific subsets of cells in blood.

Results

Qualitative analysis

The numbers of "present" and "unique" genes for each cell type are listed in Table 1. Of the 54,675 genes, higher percentages of the genes are expressed (present) by lymphocytes, including B (36.6%), CD4 (36.4%), CD8 (35.3%), and NK cells (36.2%), than by monocytes/platelets (31.9%) and PMNs (24.0%). However, there are higher percentages of unique genes for PMNs (1.2%) and monocytes/platelets (0.9%) than for lymphocytes (0–0.6%) (Table 1). Among the lymphocytes, B cells have the highest number of characteristic genes, while unique transcripts for T cells are relatively scarce due to the largely similar profiles for T cell subsets $CD4^+$ and $CD8^+$ and the profiles for NK cells.

Quantitative analysis

Among 54,675 genes (probe sets) on the array, a total of 2635 are differentially expressed between the blood cell types (parametric analysis of variance (ANOVA), $p < 0.05$ with Bonferroni correction), among which 269 are significant using a Student–Newman–Keuls post hoc test. For practical reasons, we focused on the 269-probe set list since it should contain the most characteristic genes and potentially contain genomic expression markers for each cell lineage. These 269 genes/probe sets were mathematically separated into nine clusters of relatively unique expression profiles using a hierarchical algorithm [25] as demonstrated in Fig. 1. The pattern of expression of each gene in each cluster and the fold changes of the genes are shown in separate panels on the right side of Fig. 1. In general the fold changes varied as much as 10- to 100-fold. The genes in each cluster are listed in Table 2. The left side of Fig. 1 not only shows the gene expression (y axis) for different blood cell types (x axis), but also shows the gene expression of the three individuals performed for each cell type. Note that the expression levels (red—fivefold increase; bright green—fivefold decrease) for each individual are extremely reproducible between cell types and between genes. This indicates that the microarray technological variables have a minimal effect upon the expression profiles shown in Fig. 1 and indicate that our criteria for selecting genes for each cell type are stringent and not significantly affected by individual differences at least in this preliminary study.

Table 1
Results of the qualitative analysis

Cell type	Number of present probe sets	Percentage of present probe sets	Number of unique probe sets	Percentage of unique probe sets
PMN	13,139	24.0%	155	1.2%
$CD14^+$ monocyte or platelet	17,426	31.9%	152	0.9%
$CD19^+$ B cell	20,009	36.6%	120	0.6%
$CD4^+$ T cell	19,909	36.4%	24	0.1%
$CD8^+$ T cell	19,321	35.3%	8	0.0%
$CD56^+$ NK cell	19,777	36.2%	42	0.2%

A total of 54,675 probe sets that examined approximately 39,500 genes were surveyed on each array. "Present" probe sets include probe sets that have 3 present detection calls for a specific cell type regardless of the calls for other cell types. "Unique" probe sets include probe sets that have 3 present calls for a cell type and 15 absent calls for every other cell type.

Cellular origin of blood genes regulated by neurological diseases

Fig. 2 represents a melding of the data from the current study with that from our previous disease-specific studies. The genes that were most highly regulated in ischemic stroke [8], Tourette syndrome [10], and migraine [9] were selected and the cell-specific expression of each of those genes (from the present study) is shown. As demonstrated in Fig. 2, the major genes up-regulated in whole blood after stroke were expressed mainly by PMNs and monocytes/platelets. The major genes up-regulated by Tourette syndrome were mostly from NK cells and/or $CD8^+$ T cells. The major genes up-regulated by migraine were predominantly from platelets/monocytes,

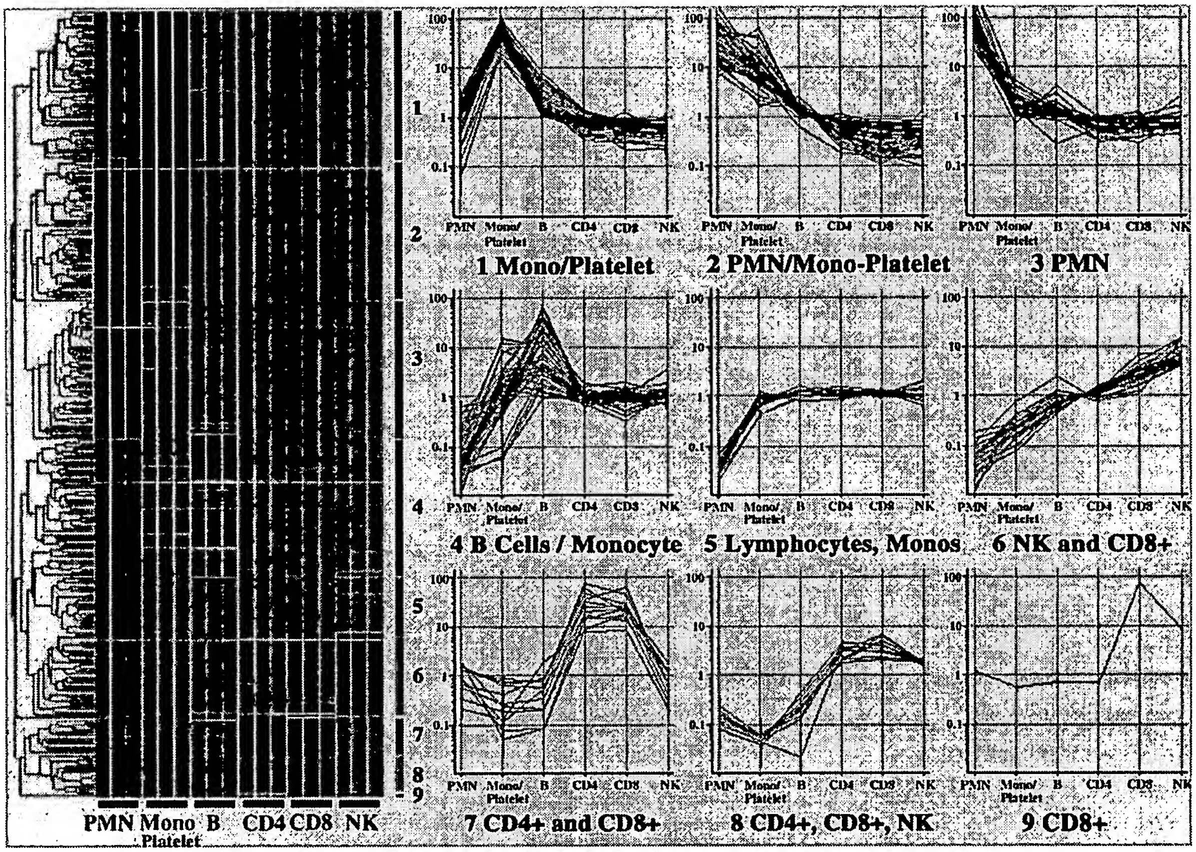


Fig. 1. A total of 269 genes that are differentially expressed between blood cell types (parametric one-way ANOVA followed by Student–Newman–Keuls post hoc test, $p < 0.05$, with Bonferroni multiple comparison correction) were subjected to a hierarchical cluster algorithm with Pearson correlation as a measure of similarity. (Left) Clusters of genes (nine clusters) with similar expression patterns are displayed from top to bottom (y axis), while cell types are displayed from left to right along the x axis. For each cell type the results of the three different individuals are shown adjacent to one another. The relative expression of each gene is color coded; red shows a fivefold increase and green shows a fivefold decrease. (Right, 1–9) Line graphs of genes segregated in the cluster analysis are shown for each of the nine clusters identified on the left. The x axis shows the cell types and the y axis shows the relative expression values (log scale) as mean −1 standard deviation (log ratio).

though there were some regulated genes from PMNs, CD4$^+$, CD8$^+$, and NK cells.

Discussion

This study surveyed the global expression profiles of six major subtypes of blood cells. These data support previous studies showing that T cells and even platelets have genes that are expressed in common, but also have genes that are fairly specific for each cell type and, perhaps more importantly, have different families of genes that tend to be expressed in a specific cell type compared to another cell type [14,18–20]. Characterization of these profiles should help elucidate the molecular and genomic basis of the development, differentiation, and function for each cell type.

Genes in cluster 1 are highly enriched in a monocyte/platelet population compared to other cell types. The recent literature shows that many genes from this cluster, such as CLU, GP1BB, PF4V1, and others (Table 2), are specifically expressed by platelets [18,20]. Cluster 2 represents genes enriched in PMNs and monocytes, while genes in cluster 3 are expressed exclusively by PMNs. Many genes in these two clusters play crucial roles in innate immunity. These include receptor molecules such as TREM1 [26], FPRL1 [27], and TLR2 [28], which are involved in microbial recognition and lead to phagocyte activation and the amplification of the inflammatory response. There are effector molecules such as MMP9 [29]; S100 proteins P, A9, and A12 [30]; and neutrophil cytosolic factors 1, 2, and 4, which participate in the neutralization of and aid clearance of microorganisms and foreign materials, and scavenger molecules such as IL1R2 [31] and TNFRSF10 [32] that help suppress excessive and harmful innate immune responses. In comparison, genes down-regulated in PMNs (cluster 5) did not provide many functional insights. The low expression of several ribosomal proteins and transcription elongation factor in this cluster may indicate a slower rate of protein translation in PMNs and is consistent with somewhat fewer RNA transcripts in this cell type (Table 1).

Several molecules expressed by B cells (cluster 4) serve important central roles in B cell development, proliferation, and differentiation, such as MS4A1 [33], BLNK [34], and BANK1 [35]. Other molecules, including immunoglobulins and HLA antigens, important for normal B cell functions, were also expressed (Table 2). While there are a few common genes between NK cells and T cells, most notably T cell receptor subunits and lymphocyte-specific tyrosine kinase (LCK)

Table 2
Results of the quantitative analysis

	Common	GenBank	Description
Cluster 1	a1/3GTP	AI972498	Clone IMAGE:4812754, mRNA
	ACRBP	AB051833	Acrosin-binding protein
	ARHGAP6	NM_001174	Rho GTPase-activating protein 6
	C21orf7	NM_020152	Chromosome 21 open reading frame 7
	CA2	M36532	Carbonic anhydrase II
	CD163	NM_004244	CD163 antigen
	CD36	NM_000072	CD36 antigen (collagen type I receptor, thrombospondin receptor)
	CD9	NM_001769	CD9 antigen (p24)
	CLEC2	NM_016509	C-type lectin-like receptor-2
	CLU	M25915	Clusterin
	CSPG2	BF590263	Chondroitin sulfate proteoglycan 2 (versican)
	CXCL5	AK026546	Chemokine (C-X-C motif) ligand 5
	CYP1B1	NM_000104	Cytochrome P450, family 1, subfamily B, polypeptide 1
	ELOVL7	AW138767	Hypothetical protein FLJ23563
	EMS1	NM_005231	EMS1 sequence (mammary tumor and squamous cell carcinoma-associated (p80/85 Src substrate)
	F13A1	NM_000129	Coagulation factor XIII, A1 polypeptide
	FSTL1	BC000055	Follistatin-like 1
	GNG11	NM_004126	Guanine nucleotide binding protein (G protein), γ11
	GP1BB	NM_000407	Glycoprotein Ib (platelet), β polypeptide
	HIST1H3H	NM_003536	Histone 1, H3h
	ITGB3	M35999	Integrin, β3 (platelet glycoprotein IIIa, antigen CD61)
	KIAA0626	NM_021647	
	MS4A6A	NM_022349	Membrane-spanning 4-domains, subfamily A, member 6A
	MYLK	AA526844	MSTP083 mRNA, complete cds
	PF4	NM_002619	Platelet factor 4 (chemokine (C-X-C motif) ligand 4)
	PF4V1	NM_002620	Platelet factor 4 variant 1
	PPBP	R64130	Proplatelet basic protein (chemokine (C-X-C motif) ligand 7)
	PRKAR2B	NM_002736	Protein kinase, cAMP-dependent, regulatory, type II, β
	PROS1	NM_000313	Protein S (α)
	PTGS1	S36219	Prostaglandin–endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
	RIN2	AL136924	Ras and Rab interactor 2

Table 2 (*continued*)

	Common	GenBank	Description
Cluster 1	SDPR	NM_004657	Serum deprivation response (phosphatidylserine binding protein)
	SDPR	BF982174	Serum deprivation response (phosphatidylserine binding protein)
	SPARC	NM_003118	Secreted protein, acidic, cysteine-rich (osteonectin)
	THBS1	BF055462	Thrombospondin 1
	TREML1	AF534823	Triggering receptor expressed on myeloid cells-like 1
	TUBB1	NM_030773	Tubulin, β1
Cluster 2	ANXA3	M63310	Annexin A3
	APOBEC3A	U03891	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A
	AQP9	NM_020980	Aquaporin 9
	BASP1	NM_006317	Brain abundant, membrane attached signal protein 1
	CD14	NM_000591	CD14 antigen
	CLECSF12	AF400600	C-type (calcium-dependent, carbohydrate-recognition domain) lectin, superfamily member 12
	CLECSF9	BC000715	C-type (calcium-dependent, carbohydrate-recognition domain) lectin, superfamily member 9
	CREB5	AI689210	cAMP-responsive element binding protein 5
	CSF3R	NM_000760	Colony-stimulating factor 3 receptor (granulocyte)
	DKFZP434B044	AL136861	Hypothetical protein DKFZp434B044
	DKFZp434H2111	AK026776	Hypothetical protein DKFZp434H2111
	FCGR2A	NM_021642	Fc fragment of IgG, low affinity IIa, receptor for (CD32)
	FLJ20273	NM_019027	RNA-binding protein
	FLJ23091	AL534095	Putative NF-κB activating protein 373
	FLJ23091	AA775681	Putative NF-κB activating protein 373
	FLJ23153	AA650281	Likely ortholog of mouse tumor necrosis-α-induced adipose-related protein
	FOS	BC004490	v-Fos FBJ murine osteosarcoma viral oncogene homolog
	FPR1	NM_002029	Formyl peptide receptor 1
	GALNAC4S-6ST	NM_014863	
	GPR86	NM_023914	G-protein-coupled receptor 86
	HIST2H2BE	NM_003528	Histone 2, H2be
	HSPC159	AK025603	HSPC159 protein
	IL13RA1	NM_001560	Interleukin 13 receptor, α1
	IL1RN	U65590	
	MNDA	NM_002432	Myeloid cell nuclear differentiation antigen

Table 2 (*continued*)

	Common	GenBank	Description
Cluster 2	NCF1	NM_000265	Neutrophil cytosolic factor 1 (47 kDa, chronic granulomatous disease, autosomal 1)
	NCF2	BC001606	Neutrophil cytosolic factor 2 (65 kDa, chronic granulomatous disease, autosomal 2)
	NCF4	NM_013416	Neutrophil cytosolic factor 4, 40 kDa
	NFE2	L13974	Nuclear factor (erythroid-derived 2), 45 kDa
	PADI4	NM_012387	Peptidyl arginine deiminase, type IV
	QPCT	NM_012413	Glutaminyl-peptide cyclotransferase (glutaminyl cyclase)
	RGS18	AF076642	Regulator of G-protein signaling 18
	S100A12	NM_005621	S100 calcium-binding protein A12 (calgranulin C)
	S100A9	NM_002965	S100 calcium-binding protein A9 (calgranulin B)
	SGK	NM_005627	Serum/glucocorticoid regulated kinase
	SLC22A4	NM_003059	Solute carrier family 22 (organic cation transporter), member 4
	SNCA	BG260394	Synuclein, α (non-A4 component of amyloid precursor)
	TLR2	NM_003264	Toll-like receptor 2
	TLR4	U93091	
	TLR8	AW872374	
	TM6SF1	NM_023003	Transmembrane 6 superfamily member 1
	TMG4	BF905445	Transmembrane γ-carboxyglutamic acid protein 4
	TREM1	NM_018643	Triggering receptor expressed on myeloid cells 1
Cluster 3	ABCA1	NM_005502	ATP-binding cassette, subfamily A (ABC1), member 1
	ACSL1	NM_001995	Acyl-CoA synthetase long-chain family member 1
	ADM	NM_001124	Adrenomedullin
	C4BPA	NM_000715	Complement component 4 binding protein, α
	CCR3	NM_001837	Chemokine (C-C motif) receptor 3
	CHI3L1	M80927	Chitinase 3-like 1 (cartilage glycoprotein-39)
	CKLFSF2	AA778552	Chemokine-like factor superfamily 2
	CYP4F3	NM_000896	Cytochrome P450, family 4, subfamily F, polypeptide 3
	EMR3	AF239764	EGF-like module-containing, mucin-like, hormone receptor-like 3
	G0S2	NM_015714	Putative lymphocyte G0/G1 switch gene

Table 2 (*continued*)

	Common	GenBank	Description
Cluster 3	GPR109B	NM_006018	Putative chemokine receptor
	HAL	NM_002108	Histidine ammonia-lyase
	IL1R2	U64094	Human soluble type II interleukin-1 receptor mRNA, complete cds
	IL8	NM_000584	Interleukin 8
	IL8RB	NM_001557	Interleukin 8 receptor, β
	KCNJ15	D87291	Potassium inwardly rectifying channel, subfamily J, member 15
	KCNJ2	BF111326	Potassium inwardly rectifying channel, subfamily J, member 2
	KRT23	NM_015515	Keratin 23 (histone deacetylase inducible)
	MANSC1	NM_018050	Hypothetical protein FLJ10298
	MGAM	NM_004668	Maltase–glucoamylase (α-glucosidase)
	MME	AI433463	Membrane metalloendopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10)
	MMP9	NM_004994	Matrix metalloproteinase 9 (gelatinase B, 92-kDa gelatinase, 92-kDa type IV collagenase)
	MSCP	BG251467	Mitochondrial solute carrier protein
	PBEF1	BC020691	Pre-B-cell colony enhancing factor 1
	PROK2	AF182069	Prokineticin 2
	PTGS2	NM_000963	Prostaglandin–endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
	S100P	NM_005980	S100 calcium-binding protein P
	SEC14L1	AI017770	SEC14-like 1 (*Saccharomyces cerevisiae*)
	TNFAIP6	NM_007115	Tumor necrosis factor, α-induced protein 6
	TNFRSF10C	AF012536	Tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain
	VNN3	NM_018399	Vanin 3
Cluster 4	AKAP2	BG540494	Paralemmin 2
	ANXA2	NM_004039	Annexin A2
	ATP1B3	U51478	ATPase, Na^+/K^+ transporting, β3 polypeptide
	BANK1	NM_017935	B-cell scaffold protein with ankyrin repeats 1
	BLNK	NM_013314	B-cell linker
	CCDC6	AK024913	cDNA: FLJ21260 fis, clone COL01441
	CPVL	NM_031311	Carboxypeptidase, vitellogenic-like
	CXXC5	BC006428	CXXC finger 5
	DPYSL2	NM_001386	Dihydropyrimidinase-like 2
	FCRH3	BF514552	Fc receptor-like protein 3

(continued on next page)

Table 2 (*continued*)

	Common	GenBank	Description
Cluster 4	FLJ20668	AI707896	Hypothetical protein FLJ20668
	HLA-DPA1	M27487	Major histocompatibility complex, class II, DP α1
	HLA-DPB1	NM_002121	Major histocompatibility complex, class II, DP β1
	HLA-DRB3	AJ297586	Major histocompatibility complex, class II, DR β3
	ICSBP1	AI073984	Interferon consensus sequence binding protein 1
	IGLJ3	X57812	Immunoglobulin λ joining 3
	KYNU	D55639	Kynureninase (L-kynurenine hydrolase)
	LIPA	NM_000235	Lipase A, lysosomal acid, cholesterol esterase (Wolman disease)
	MGC27165	S55735	Hypothetical protein MGC27165
	MS4A1	BC002807	Membrane-spanning 4-domains, subfamily A, member 1
	MS4A7	AI301935	Membrane-spanning 4-domains, subfamily A, member 7
	NAP1L	AI763426	Napsin B pseudogene
	P2RX5	U49396	Purinergic receptor P2X, ligand-gated ion channel, 5
	POU2AF1	NM_006235	POU domain, class 2, associating factor 1
	PRDX4	NM_006406	Peroxiredoxin 4
	SPAP1	AL833361	SH2 domain-containing phosphatase anchor protein 1
	TCF4	BF592782	Transcription factor 4
	TPD52	AA524023	Tumor protein D52
	TRAF5	NM_004619	TNF receptor-associated factor 5
	TXNDC5	NM_030810	Thioredoxin domain-containing 5
	VAMP8	NM_003761	Vesicle-associated membrane protein 5 (myobrevin)
Cluster 5	C1QBP	L04636	Complement component 1, q subcomponent binding protein
	CLNS1A	AF005422	Chloride channel, nucleotide-sensitive, 1A
	DOCK10	NM_017718	
	FLJ11171	AK023183	Hypothetical protein FLJ11171
	FLJ20160	AA133311	FLJ20160 protein
	FLJ38426	BF679966	Hypothetical protein FLJ38426
	LRPPRC	AI653608	Leucine-rich PPR-motif-containing
	MGC5395	BG287862	Hypothetical protein MGC5395
	MRPL3	BC003375	Mitochondrial ribosomal protein L3
	MRPL9	BC004517	Mitochondrial ribosomal protein L9
	MRPS23	BC000242	Mitochondrial ribosomal protein S23
	NDUFB2	NM_004546	NADH dehydrogenase (ubiquinone) 1β subcomplex, 2, 8 kDa
	PLAC8	NM_016619	Placenta-specific 8

Table 2 (*continued*)

	Common	GenBank	Description
Cluster 5	PP	NM_021129	Pyrophosphatase (inorganic)
	RAFTLIN	D42043	Raft-linking protein
	RPL10A	NM_007104	Ribosomal protein L10a
	SUCLG2	AL050226	Succinate-CoA ligase, GDP-forming, β subunit
	TCERG1	NM_006706	Transcription elongation regulator 1 (CA150)
	VPS45A	AF165513	Vacuolar protein sorting 45A (yeast)
	AKR1C3	AB018580	Aldo-keto reductase family 1, member C3 (3-α hydroxysteroid dehydrogenase, type II)
	CD160	NM_007053	CD160 antigen
	CD3Z	J04132	CD3Z antigen, ζ polypeptide (TiT3 complex)
	EAT2	BC022407	SH2 domain-containing molecule EAT2
Cluster 6	GNLY	M85276	*Homo sapiens* NKG5 gene, complete cds
	GPR56	AL554008	G-protein-coupled receptor 56
	GZMB	J03189	Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)
	GZMH	M36118	Granzyme H (cathepsin G-like 2, protein h-CCPX)
	IFIX	AI827431	Interferon-inducible protein X
	KLRC3	NM_002261	Synonyms: NKG2E, NKG2-E
	KLRD1	U30610	Killer cell lectin-like receptor subfamily D, member 1
	KLRF1	NM_016523	Killer cell lectin-like receptor subfamily F, member 1
	KLRK1	AF439512	Killer cell lectin-like receptor subfamily K, member 1
	KSP37	AB021123	Ksp37 protein
	MGC61571	BE963026	Hypothetical protein MGC61571
	NKG7	NM_005601	Natural killer cell group 7 sequence
	SAMD3	AI129628	Sterile α motif domain-containing 3
	SPON2	NM_012445	Spondin 2, extracellular matrix protein
	SPUVE	NM_007173	Protease, serine, 23
	TGFBR3	NM_003243	Transforming growth factor, β receptor III (betaglycan, 300 kDa)
	TRD@	X06557	T-cell receptor δ locus
		AA227879	Transcribed sequences
Cluster 7	C6orf190	BC043608	Chromosome 6 open reading frame 207, mRNA (cDNA clone IMAGE: 5764019), partial cds

Table 2 (*continued*)

	Common	GenBank	Description
Cluster 7	CCR7	NM_001838	Chemokine (C-C motif) receptor 7
	CD28	NM_006139	CD28 antigen (Tp44)
	GZMK	NM_002104	Granzyme K (serine protease, granzyme 3; tryptase II)
	IL7R	NM_002185	Interleukin 7 receptor
	LEF1	AF288571	Lymphoid enhancer-binding factor 1
	MAL	NM_002371	Mal, T-cell differentiation protein
	NELL2	NM_006159	NEL-like 2 (chicken)
	RGS1	S59049	Regulator of G-protein signaling 1
	TRIM	AJ240085	T-cell receptor-interacting molecule
		M12959	Human mRNA for T-cell receptor α chain
Cluster 8	BCL11B	AA918317	B-cell CLL/lymphoma 11B (zinc finger protein)
	CD2	NM_001767	CD2 antigen (p50), sheep red blood cell receptor
	H963	NM_013308	Platelet-activating receptor homolog
	LCK	NM_005356	Lymphocyte-specific protein tyrosine kinase
	LOC283666	AW006185	Hypothetical protein LOC283666, mRNA (cDNA clone IMAGE: 4415549), partial cds
	TRGV9	M30894	T-cell receptor γ locus
	TRGV9	M16768	T-cell receptor (V-J-C) precursor;
		BC040965	Clone IMAGE:5747561, mRNA
		AF043179	*H. sapiens* T-cell receptor β chain (TCRBV13S1–TCRBJ2S1) mRNA, complete cds
		AL559122	T-cell receptor β chain BV20S1 BJ1-5 BC1 mRNA, complete cds
		M15564	T-cell receptor precursor; human T-cell receptor rearranged β-chain V-region (V-D-J) mRNA
Cluster 9	CD8A	AW006735	CD8 antigen, α polypeptide (p32)

A total of 269 genes that are differentially expressed between blood cell types (parametric one-way ANOVA followed by Student–Newman–Keuls post hoc test, $p < 0.05$, with Bonferroni multiple correction) were segregated into nine clusters using a hierarchical cluster algorithm. Cluster 1, specifically expressed by monocytes/platelets. Cluster 2, highly expressed by PMNs and, to a lesser degree, monocytes/platelets. Cluster 3, specifically expressed by PMNs. Cluster 4, highly expressed by B cells and, to a lesser degree, monocytes/platelets. Cluster 5, expressed by all cell types except PMNs. Cluster 6, highly expressed by NK cells and, to a lesser degree, $CD8^+$ T cells. Cluster 7, highly expressed by $CD4^+$ and $CD8^+$ T cells. Cluster 8, highly expressed by $CD4^+$ and $CD8^+$ T cells and NK cells. Cluster 9, highly expressed by $CD8^+$ T cells and NK cells. Note that multiple probe sets for the same gene are presented only once.

(cluster 8), NK cells can be mostly distinguished by the higher expression of genes including NKG5, NKG7, NKG2E, KLRD, and KLRF1 (cluster 6). Interestingly, genes originally cloned from cytotoxic T cells such as GZMB and GZMH (granzyme B and H) have a higher expression in NK cells but are also present in $CD8^+$ T cells (Figs. 1 and 2; Table 2). The overall genomic patterns of $CD4^+$ and $CD8^+$ T cells are very similar and can be distinguished from other cell types by well-known T cell markers such as CD28 [36], IL7R [37], the chemokine receptor CCR7 [38], and others (cluster 7). Moreover, a novel gene, NELL2 [39], that is abundant in neural tissues is also highly expressed by both $CD4^+$ and $CD8^+$ T cells. Not surprisingly, CD8 antigen distinguishes $CD4^+$ and $CD8^+$ T cells (cluster 9). In a recent study of T cells purified from buffy coats from healthy donors, approximately 50% of the genes in clusters 7 and 8 in this study were present (6/11 and 5/11, respectively) [14], suggesting that these sets of genes are consistent even with different purification methodologies and different individuals.

Several limitations of the current study need to be emphasized. The purification of most of the cell types was less than desirable. However, it was for this reason that we identified gene clusters that are quite different from each other and then examined how these gene clusters related to the imperfectly separated cell types in blood (Fig. 1). Using this approach we can say that the genes or gene clusters that are expressed only for a given cell type are in fact expressed by that cell type. Genes that appear to be expressed by two or more cell types might be shared between those cell types or could be expressed by only one cell type but could not be differentiated because of imprecise separation of cells. In addition, a given cell type is likely to have heterogeneous subtypes, and thus there are likely to be different gene expression signatures for these subtypes. A good example is $CD4^+$ cells, among which are Th1 and Th2 $CD4^+$ cells, which have very different functions in the immune system [40] and which were not differentiated in this study. Moreover, the increased expression by any given cell type could be due to a number of different factors including the numbers of cells. This is not likely to be a major factor since the changes in gene expression shown usually vary from 5- to 100-fold, and it is unlikely that the numbers of these types of cells vary this much.

Other limitations of the results include the negative selection method of isolating the cells. Even though the selected cells do not undergo antibody binding used for positive selection, the negative selection likely activates complement that could affect the cells being surveyed. In addition, because the negative selection method does not produce pure populations of cells, it is possible that the contaminating cells activate the primary cells being isolated. Therefore, any given profile for each cell type could represent some degree of activation of the cells. Since the data at least for the three individuals examined look fairly consistent, if there is nonspecific activation of cells with negative selection at least it is fairly consistent from one experiment to the next.

An additional limitation of the current study was the inability to distinguish gene expression by platelets from gene expression by monocytes because of the negative selection methods used.

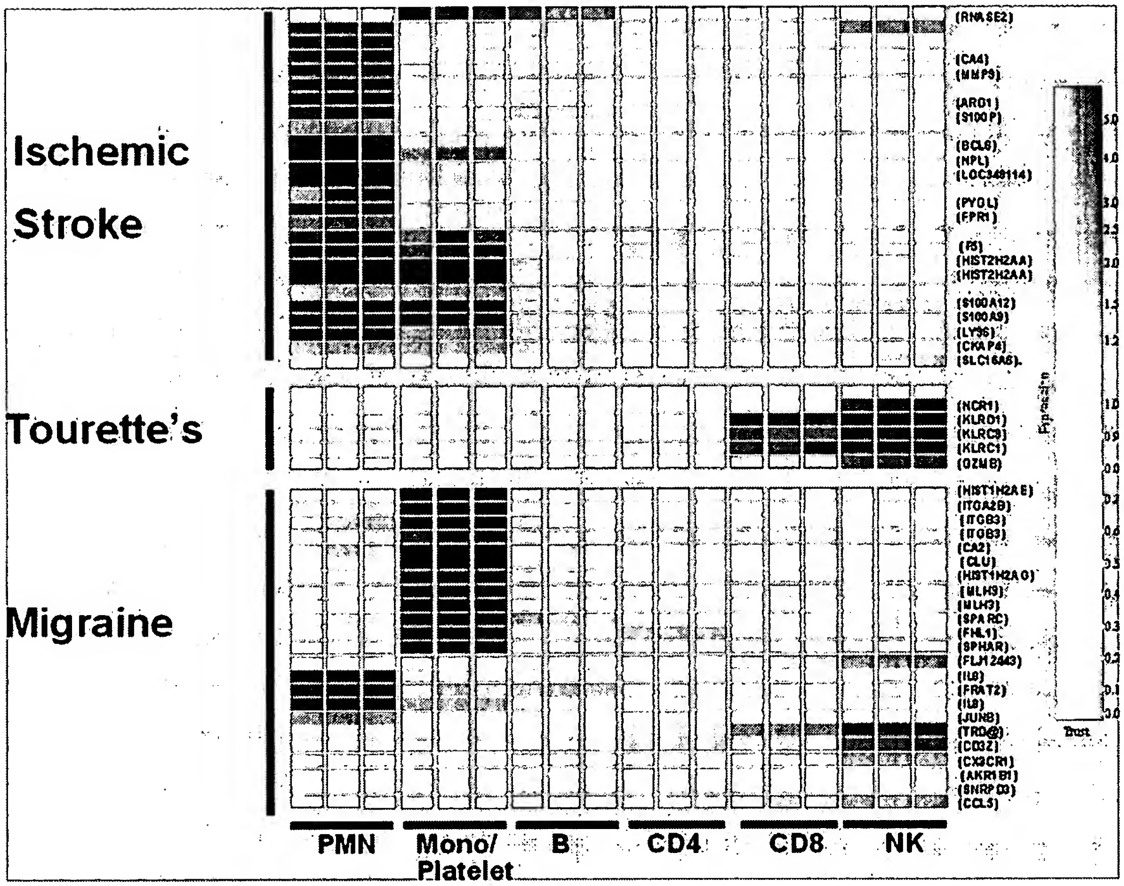


Fig. 2. Cellular origins of genes that have previously been shown to be regulated by ischemic stroke [8], Tourette syndrome [10], and migraine [9]. These genes were identified from RNA isolated from whole blood by comparing to healthy controls and patients with other diseases. Genes regulated by each disease are listed from top to bottom (y axis) and their relative expression in each blood cell type is color coded and displayed from left to right (x axis). The list of displayed genes from the top down for each disorder follows and the genes are numbered and named according to the Affymetrix probe sets: ischemic stroke, 206111_at (RNASE2), 239893_at, 234632_x_at, 206209_s_at (CA4), 203936_s_at (MMP9), 244218_at, 206177_s_at (ARG1), 204351_at (S100P), 232595_at, 228758_at (BCL6), 240440_at (NPL), 225899_x_at (LOC349114), 227129_x_at, 232958_at (PYGL), 205118_at (FPR1), 228642_at, 231029_at, 204714_s_at (F5), 214290_s_at (HIST2H2AA), 218280_x_at (HIST2H2AA), 222303_at, 205863_at (S100A12), 203535_at (S100A9), 206584_at (LY96), 200999_s_at (CKAP4), 207038_at (SLC16A6); Tourette syndrome, 212775_at (KIAA0657), 207860_at (NCR1), 207796_x_at (KLRD1), 207723_s_at (KLRC3), 206785_s_at (KLRC1), and 210164_at (GZMB); Migraine, 214469_at (HIST1H2AE), 206494_s_at (ITGA2B), 204627_s_at (ITGB3), 216261_at (ITGB3), 209301_at (CA2), 208791_at (CLU), 207156_at (HIST1H2AG), 217216_x_at (MLH3), 204838_s_at (MLH3), 200665_s_at (SPARC), 210299_s_at (FHL1), 206272_at (SPHAR), 201818_at (FLJ12443), 211506_s_at (IL8), 209864_at (FRAT2), 202859_x_at (IL8), 201473_at (JUNB), 217143_s_at (TRD@), 210031_at (CD3Z), 205898_at (CX3CR1), 201272_at (AKR1B1), 202567_at (SNRPD3) 204655_at (CCL5).

However, gene expression profiles for platelets have been published [18,20]. It is notable that platelets lack a nucleus and nuclear DNA, and hence the mRNA found in platelets is derived from the megakaryocytes that form the platelets. A similar situation exists for red blood cells, which contain mRNA but have no nucleus or nuclear DNA. The importance of differentiating platelet from monocyte RNAs is emphasized by the data in Fig. 2. Even though stroke and migraine appear to express genes in both platelets and monocytes, careful examination of the gene lists for both shows that in stroke the genes induced are mainly in monocytes, and in migraine the genes are induced mainly in platelets (see below). It is certainly possible that a genetic disease like migraine could be associated with changes in gene expression in platelets and not other cellular elements in the blood. This is emphasized by a number of clinical studies in migraine as mentioned next.

Finally, it is important to reemphasize that the data shown in this study are for expression in three normal, healthy individuals, and the data for the three diseases were obtained by comparing the published disease-regulated genes to the genes expressed by different cell types in the healthy persons in this study. The expression profiles for the individual cell types shown here can be viewed only as being preliminary, since only three individuals were studied. Large numbers of individuals may be necessary to derive reliable gene profiles for individual cell types in blood, since age, gender, race, genetic background, lifestyle, diet, concurrent diseases and medications, and many other factors are likely to influence cell type-related gene expression. The current study does, however, emphasize the need for future studies to isolate these cell types in individuals with each disease and replicate the gene expression profiles for each cell type in each

individual with each disease. An unexpected benefit to isolating individual cell types was that the fold differences of gene expression is 10- to 100-fold, compared to fold changes in whole blood, at least in neurological diseases, on the order of 2- to 3-fold [6–10,13–17,41]. The ability to detect high fold changes in these cell-specific data could be due in part to removal of high-abundance RNAs that populate whole blood and in part to using an approach that highlights cell differences rather than averaging them.

The data shown suggest that the cell type-specific data could serve as a powerful guide to understanding the relative contribution of each cell type to the overall gene expression profile caused by various diseases. The data show that different neurological diseases affect gene expression through distinct blood cell populations. Tourette syndrome, a neuropsychiatric tic disorder that has been proposed to be caused by an autoimmune response to streptococcus at least in some patients [42,43], appears to be associated with changes in gene expression in NK cells and/or $CD8^+$ cells based upon the results of this study and our previous findings [10]. In contrast, the blood genomic response following acute ischemic stroke was predominantly from PMNs based upon the current findings and those of our recent ischemic stroke study [8]. This agrees with human and animal studies showing that polymorphonuclear cells are the major cell type that initially infiltrate areas of cerebral infarction following stroke and appear to play a major role in pathogenesis [44–46]. Importantly, infection and predisposition to inflammation may be risk factors for stroke [47]. In comparison, the blood genomic pattern for migraine headache is more heterogeneous, with several blood cell types affected, including platelets and monocytes. Platelets and abnormalities of serotonin in platelets have long been implicated in the pathogenesis of migraine and its genetic basis and may contribute to an increased risk of stroke [48–51]. These data suggest that although neuroinflammation plays a crucial role in the pathological process of both cerebral ischemia [52] and migraine [53], the involved blood cells and genes are distinct. This further underscores the importance of identifying the blood cell types associated with a given disorder, to formulate mechanistic hypotheses and accurately characterize surrogate blood markers for diagnostic, prognostic, and treatment purposes.

Materials and methods

Separation of blood cell subtypes

Blood was drawn from three healthy donors for all cell subsets. The donors were healthy, ages 32, 33, and 59; two were male; and none had any concurrent infection or major medical illnesses. Subsets of mononuclear cells including $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, $CD56^+$ NK cells, and $CD14^+$ monocytes were enriched using the RosetteSep negative selection method (StemCell Technologies, BC, Canada). This method was chosen as it provided a short processing time and the desired cell types remain largely undisturbed. This method thus helped to minimize potential alterations in gene expression due to excessive handling during the cell isolation and to avoid possible effects of positive selection of cells on gene expression [21,22]. For each cell subtype, 8 ml whole blood was drawn into Vacutainer CPT cell preparation tubes that contained sodium citrate and Ficoll (Becton–Dickinson, NJ, USA). Four hundred microliters of RosetteSep antibody cocktail was added to each tube and incubated for 20 min at room temperature. The antibody cocktail cross-links unwanted cells in whole blood to red blood cells, forming immunorosettes with increased density. After incubation, the blood collected in the CPT tubes was centrifuged for 20 min at 1800 g to precipitate the unwanted cells and precipitate free RBCs. The desired cells, not labeled with antibody, were collected from the plasma:Ficoll interface and washed once with PBS and the RNA was isolated using Trizol reagent. The purity of cells separated with this protocol was 90 ± 5% for $CD4^+$ T cells, 76 ± 8% for $CD8^+$ T cells, 81 ± 8% for $CD19^+$ B cells, 74 ± 10% for $CD56^+$ NK cells, and 69 ± 12% for $CD14^+$ monocytes (mean ± SD) according to confirmatory flow cytometry data provided by the manufacturer.

It is noted that platelet contamination has been reported for $CD14^+$ monocytes using the RosetteSep enrichment method. Therefore, the "monocyte" gene expression pattern obtained in this study may come from both monocytes and platelets and is therefore referred to as "mono/platelet" in the text. However, importantly, platelet contamination is not seen in lymphocyte enrichments as the lymphocyte enrichment cocktails contain anti-CD36, which removes platelets along with monocytes.

For the separation of PMNs, 6 ml sodium citrate-anti-coagulated venous blood was carefully layered on 3 ml leukocyte separation media (Histopaque-1119 and Histopaque-1077; Sigma–Aldrich, St. Louis, MO, USA). After a 700*g* centrifugation for 30 min at room temperature, the "mononuclear" layer together with fluid within 0.5 cm of this layer was aspirated and discarded, while the "granulocyte" layer was transferred to a new tube and washed once with phosphate-buffered saline. The purity of the PMNs obtained was ~98% based on microscopic examination after Wright–Giemsa staining.

RNA isolation

The collected pellets of each cell subtype were immediately homogenized with Trizol reagent (Invitrogen, Carlsbad, CA, USA). The aqueous phase containing RNA was separated by centrifugation after adding chloroform. RNA was recovered by precipitation with isopropyl alcohol and washed with 75% ethanol. RNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the quality was assessed using an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA, USA). We processed RNA only when the A_{260}/A_{280} absorbance ratio of the cleaned RNA exceeded 2.0 and 28S/18S ratio equaled or exceeded 1.8 for these microarray studies.

Microarray processing

For each blood cell subtype ($CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, $CD56^+$ NK cells, $CD14^+$ monocytes, and PMNs), 25 ng total RNA was labeled using a two-cycle target labeling protocol and hybridized to arrays. A total of 36 (6 arrays for each cell type, for three subjects, with technical duplicates done for each) Affymetrix Human 2.0 Plus arrays were used (Affymetrix, Santa Clara, CA, USA) in this study, with each array containing 54,675 probe sets referred to as such or as "genes" in the text. Six arrays for each cell type were used for each of the three subjects (n = 18 arrays), and technical duplicates of these were then processed (n = 36 arrays total). The probe sets were on one chip that surveyed 47,000 transcripts from ~39,500 potential human genes (Affymetrix Technical Manual). Sample labeling, hybridization to chips, and image scanning were performed according to the Affymetrix *Expression Analysis Technical Manual.*

Data analysis

After the arrays were scanned, the raw expression values (probe level data) for each gene were saved in Affymetrix.cel and Affymetrix.dat files. The probe level data were then collated using GC-Robust Multi-array Average (GCRMA-EB) software (http://www.bioconductor.org/). This involved nonlinear background reduction, quantile normalization, and summarization by median polishing [23,24]. The technical replicates were averaged,

and therefore the analyses were performed on 18 independent data sets (three individuals and six different cell types for each individual). We used both qualitative and quantitative methods to assess the expression signatures of each cell type.

Qualitative method

This method was used to determine genes that are uniquely expressed by each cell type. Affymetrix GCOS software was used to generate "detection calls," i.e., "present," "absent," or "marginal," for each gene on each array based on Wilcoxon's signed rank test (http://www.affymetrix.com/support/technical/technotes/statistical_reference_guide.pdf). Briefly, the present genes represent transcripts that can be clearly detected by arrays, while the absent calls are generally given to transcripts that are below the detection threshold and cannot be reliably distinguished from noise. We determined the number of *genes that are present in each cell type* (with 3 present calls in three arrays regardless of calls from other cell types) and the number of *genes that are present only in each specific cell type* (with 3 present calls in three arrays and 15 absent calls in all other cell types). This method tends to eliminate genes that are marginally associated with a given cell type. As noted above, the technical replicates were averaged together for this qualitative analysis and the quantitative analysis that follows.

Quantitative methods

The probe level data from .cel files were first collated using Robust Multi-array Average (RMA) software (http://www.bioconductor.org/). After nonlinear background reduction and quantile normalization and summarization, probe level data were generated by median polishing each gene to produce a quantitative expression assessment in each cell sample [23,24]. Genes that were differentially expressed among cell subtypes were identified using a parametric one-way ANOVA followed by the Student–Newman–Keuls post hoc test with subtype as the variable (GENESPRING 7 software; Silicon Genetics, Redwood City, CA, USA). The type I error probability value (p value) was corrected with the Bonferroni method for multiple test comparisons and $p < 0.05$ was considered significant. The identified genes were subjected to a hierarchical cluster analysis using Pearson correlation as the similarity measure (Fig. 1).

Cellular origin of genes regulated by ischemic stroke, migraine, and Tourette syndrome

The genes up-regulated by ischemic stroke, migraine, and Tourette syndrome were identified in three previous studies by comparing these conditions to healthy controls and patients with other neurological disorders. RNA isolated from whole blood was used for all of these previous studies. The ischemic stroke study was performed using U133 2.0 Plus arrays [8], while the Tourette and migraine studies were carried out using human U95Av 2 arrays [9,10]. For the ischemic stroke study 45 stroke samples were compared to 14 healthy control samples [8]. For the migraine headache study 22 patients with migraine were compared to 56 control samples from patients with a variety of other disorders [9]. For the Tourette syndrome study, 16 patients with Tourette syndrome who had at least one first degree relative with Tourette syndrome were compared to 113 samples from control patients with a variety of other disorders including epilepsy and headache and healthy controls [10]. With the current subtype-specific expression data, we attempted to address the cell types that are affected by each disease. To make a direct comparison, genes from Affymetrix U95Av 2 arrays were matched to genes on the Affymetrix U133 2.0 Plus arrays through the Netaffx database (www.affymetrix.com) and their expression across different blood cell types was examined and visualized (Fig. 2).

Acknowledgments

This study was supported by National Institutes of Health Grants NS043252, NS 028167, NS042774, and NS044283 (F. R.S.). This work was partially supported by the MIND Institute Genomics Core and Grant PO1 ES 11269 from the NIEHS and U.S. EPA.

References

[1] T.R. Golub, D.K. Slonim, P. Tamayo, C. Huard, M. Gaasenbeek, J.P. Mesirov, H. Coller, M.L. Loh, J.R. Downing, M.A. Caligiuri, C.D. Bloomfield, E.S. Lander, Molecular classification of cancer: class discovery and class prediction by gene expression monitoring, Science 286 (1999) 531–537.

[2] A.A. Alizadeh, M.B. Eisen, R.E. Davis, C. Ma, I.S. Lossos, A. Rosenwald, J.C. Boldrick, H. Sabet, T. Tran, X. Yu, J.I. Powell, L. Yang, G.E. Marti, T. Moore, J. Hudson Jr., L. Lu, D.B. Lewis, R. Tibshirani, G. Sherlock, W.C. Chan, T.C. Greiner, D.D. Weisenburger, J.O. Armitage, R. Warnke, L.M. Staudt, et al., Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling, Nature 403 (2000) 503–511.

[3] L. Bennett, A.K. Palucka, E. Arce, V. Cantrell, J. Borvak, J. Banchereau, V. Pascual, Interferon and granulopoiesis signatures in systemic lupus erythematosus blood, J. Exp. Med. 197 (2003) 711–723.

[4] R.A. Heller, M. Schena, A. Chai, D. Shalon, T. Bedilion, J. Gilmore, D.E. Woolley, R.W. Davis, Discovery and analysis of inflammatory disease-related genes using cDNA microarrays, Proc. Natl. Acad. Sci. USA 94 (1997) 2150–2155.

[5] Y. Tang, A. Lu, B.J. Aronow, F.R. Sharp, Blood genomic responses differ after stroke, seizures, hypoglycemia, and hypoxia: blood genomic fingerprints of disease, Ann. Neurol. 50 (2001) 699–707.

[6] A.R. Whitney, M. Diehn, S.J. Popper, A.A. Alizadeh, J.C. Boldrick, D.A. Relman, P.O. Brown, Individuality and variation in gene expression patterns in human blood, Proc. Natl. Acad. Sci. USA 100 (2003) 1896–2191.

[7] Y. Tang, A. Lu, R. Ran, B.J. Aronow, E.K. Schorry, R.J. Hopkin, D.L. Gilbert, T.A. Glauser, A.D. Hershey, N.W. Richtand, M. Privitera, A. Dalvi, A. Sahay, J.P. Szaflarski, D.M. Ficker, N. Ratner, F.R. Sharp, Human blood genomics: distinct profiles for gender, age and neurofibromatosis type 1, Brain Res. Mol. Brain Res. 132 (2004) 155–167.

[8] Y. Tang, H. Xu, X.L. Du, W. Walker, A. Lu, R. Ran, J.P. Gregg, M. Reilly, A. Pancioli, J.C. Khoury, L.R. Sauerbeck, J.A. Carrozzella, J. Spilker, J.F. Clark, K.R. Wagner, E.C. Jauch, P. Verro, D.J. Chang, J.P. Broderick, F.R. Sharp, Gene expression in blood changes rapidly in neutrophils and monocytes following ischemic stroke in humans: a microarray study, J. Cereb. Blood Flow Metab. (in press).

[9] A.D. Hershey, Y. Tang, S.W. Powers, M.A. Kabbouche, D.L. Gilbert, T.A. Glauser, F.R. Sharp, Genomic abnormalities in patients with migraine and chronic migraine: preliminary blood gene expression suggests platelet abnormalities, Headache 44 (2004) 994–1004.

[10] Y. Tang, D.L. Gilbert, T.A. Glauser, A.D. Hershey, F.R. Sharp, Blood gene expression profiling of neurologic diseases: a pilot microarray study, Arch. Neurol. 62 (2005) 210–215.

[11] N.C. Twine, J.A. Stover, B. Marshall, G. Dukart, M. Hidalgo, W. Stadler, T. Logan, J. Dutcher, G. Hudes, A.J. Dorner, D.K. Slonim, W.L. Trepicchio, M.E. Burczynski, Disease-associated expression profiles in peripheral blood mononuclear cells from patients with advanced renal cell carcinoma, Cancer Res. 63 (2003) 6069–6075.

[12] A.H. Iglesias, S. Camelo, D. Hwang, R. Villanueva, G. Stephanopoulos, F. Dangond, Microarray detection of E2F pathway activation and other targets in multiple sclerosis peripheral blood mononuclear cells, J. Neuroimmunol. 150 (2004) 163–177.

[13] M.S. Forrest, Q. Lan, A.E. Hubbard, L. Zhang, R. Vermeulen, X. Zhao, G. Li, Y.Y. Wu, M. Shen, S. Yin, S.J. Chanock, N. Rothman, M.T. Smith, Discovery of novel biomarkers by microarray analysis of peripheral blood mononuclear cell gene expression in benzene-exposed workers, Environ. Health Perspect. 113 (2005) 801–807.

[14] J.P. Cobb, M.N. Mindrinos, C. Miller-Graziano, S.E. Calvano, H.V. Baker, W. Xiao, K. Laudanski, B.H. Brownstein, C.M. Elson, D.L. Hayden, D.N. Herndon, S.F. Lowry, R.V. Maier, D.A. Schoenfeld, L.L. Moldawer, R.W. Davis, R.G. Tompkins, P. Bankey, T. Billiar, D. Camp, I. Chaudry, B. Freeman, R. Gamelli, N. Gibran, B. Harbrecht, W. Heagy, D. Heimbach, J.

Horton, J. Hunt, J. Lederer, J. Mannick, B. McKinley, J. Minei, E. Moore, F. Moore, R. Munford, A. Nathens, G. O'Keefe, G. Purdue, L. Rahme, D. Remick, M. Sailors, M. Shapiro, G. Silver, R. Smith, G. Stephanopoulos, G. Stormo, M. Toner, S. Warren, M. West, S. Wolfe, V. Young, Application of genome-wide expression analysis to human health and disease, Proc. Natl. Acad. Sci. USA 102 (2005) 4801–4806.

[15] Y. Tang, M.B. Schapiro, D.N. Franz, B.J. Patterson, F.J. Hickey, E.K. Schorry, R.J. Hopkin, M. Wylie, T. Narayan, T.A. Glauser, D.L. Gilbert, A. D. Hershey, F.R. Sharp, Blood expression profiles for tuberous sclerosis complex 2, neurofibromatosis type 1, and Down's syndrome, Ann. Neurol. 56 (2004) 808–814.

[16] F. Borovecki, L. Lovrecic, J. Zhou, H. Jeong, F. Then, H.D. Rosas, S. M. Hersch, P. Hogarth, B. Bouzou, R.V. Jensen, D. Krainc, Genome-wide expression profiling of human blood reveals biomarkers for Huntington's disease, Proc. Natl. Acad. Sci. USA 102 (2005) 11023–11028.

[17] D.F. Moore, H. Li, N. Jeffries, V. Wright, R.A. Cooper Jr., A. Elkahloun, M.P. Gelderman, E. Zudaire, G. Blevins, H. Yu, E. Goldin, A.E. Baird, Using peripheral blood mononuclear cells to determine a gene expression profile of acute ischemic stroke: a pilot investigation, Circulation 111 (2005) 212–221.

[18] P. Bugert, A. Dugrillon, A. Gunaydin, H. Eichler, H. Kluter, Messenger RNA profiling of human platelets by microarray hybridization, Thromb. Haemostasis 90 (2003) 738–748.

[19] F.B. Stentz, A.E. Kitabchi, Transcriptome and proteome expression in activated human CD4 and CD8 T-lymphocytes, Biochem. Biophys. Res. Commun. 324 (2004) 692–696.

[20] D.V. Gnatenko, J.J. Dunn, S.R. McCorkle, D. Weissmann, P.L. Perrotta, W.F. Bahou, Transcript profiling of human platelets using microarray and serial analysis of gene expression, Blood 101 (2003) 2285–2293.

[21] E. Elkord, P.E. Williams, H. Kynaston, A.W. Rowbottom, Human monocyte isolation methods influence cytokine production from in vitro generated dendritic cells, Immunology 114 (2005) 204–212.

[22] S. Matsuzaki, K. Shinozaki, N. Kobayashi, K. Agematsu, Polarization of Th1/Th2 in human CD4 T cells separated by CD62L: analysis by transcription factors, Allergy 60 (2005) 780–787.

[23] B.M. Bolstad, R.A. Irizarry, M. Astrand, T.P. Speed, A comparison of normalization methods for high density oligonucleotide array data based on variance and bias, Bioinformatics 19 (2003) 185–193.

[24] R.A. Irizarry, B.M. Bolstad, F. Collin, L.M. Cope, B. Hobbs, T.P. Speed, M. Astrand, Summaries of Affymetrix Genechip probe level data: a comparison of normalization methods for high density oligonucleotide array data based on variance and bias, Nucleic Acids Res. 31 (2003) e15.

[25] M.B. Eisen, P.T. Spellman, P.O. Brown, D. Botstein, Cluster analysis and display of genome-wide expression patterns, Proc. Natl. Acad. Sci. USA 95 (1998) 14863–14868.

[26] S. Knapp, S. Gibot, A. de Vos, H.H. Versteeg, M. Colonna, T. van der Poll, Cutting edge: expression patterns of surface and soluble triggering receptor expressed on myeloid cells-1 in human endotoxemia, J. Immunol. 173 (2004) 7131–7134.

[27] Y. Le, P.M. Murphy, J.M. Wang, Formyl-peptide receptors revisited, Trends Immunol. 23 (2002) 541–548.

[28] A.O. Aliprantis, R.B. Yang, M.R. Mark, S. Suggett, B. Devaux, J.D. Radolf, G.R. Klimpel, P. Godowski, A. Zychlinsky, Cell activation and apoptosis by bacterial lipoproteins through Toll-like receptor-2, Science 285 (1999) 736–739.

[29] G. Opdenakker, P.E. Van den Steen, B. Dubois, I. Nelissen, E. Van Coillie, S. Masure, P. Proost, J. Van Damme, Gelatinase B functions as regulator and effector in leukocyte biology, J. Leukocyte Biol. 69 (2001) 851–859.

[30] J. Roth, T. Vogl, C. Sorg, C. Sunderkotter, Phagocyte-specific S100 proteins: a novel group of proinflammatory molecules, Trends Immunol. 24 (2003) 155–158.

[31] E. Bourke, A. Cassetti, A. Villa, E. Fadlon, F. Colotta, A. Mantovani, IL-1 beta scavenging by the type II IL-1 decoy receptor in human neutrophils, J. Immunol. 170 (2003) 5999–6005.

[32] J.P. Sheridan, S.A. Marsters, R.M. Pitti, A. Gurney, M. Skubatch, D. Baldwin, L. Ramakrishnan, C.L. Gray, K. Baker, W.I. Wood, A.D. Goddard, P. Godowski, A. Ashkenazi, Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors, Science 277 (1997) 818–821.

[33] T.F. Tedder, M. Streuli, S.F. Schlossman, H. Saito, Isolation and structure of a cDNA encoding the B1 (CD20) cell-surface antigen of human B lymphocytes, Proc. Natl. Acad. Sci. USA 85 (1988) 208–212.

[34] C. Fu, C.W. Turck, T. Kurosaki, A.C. Chan, BLNK: a central linker protein in B cell activation, Immunity 9 (1998) 93–103.

[35] K. Yokoyama, I.H. Su, T. Tezuka, T. Yasuda, K. Mikoshiba, A. Tarakhovsky, T. Yamamoto, Bank regulates Bcr-induced calcium mobilization by promoting tyrosine phosphorylation of IP(3) receptor, EMBO J. 21 (2002) 83–92.

[36] G. Magistrelli, P. Jeannin, G. Elson, J.F. Gauchat, T.N. Nguyen, J.Y. Bonnefoy, Y. Delneste, Identification of three alternatively spliced variants of human CD28 mRNA, Biochem. Biophys. Res. Commun. 259 (1999) 34–37.

[37] S.M. Kaech, J.T. Tan, E.J. Wherry, B.T. Konieczny, C.D. Surh, R. Ahmed, Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells, Nat. Immunol. 4 (2003) 1191–1198.

[38] F. Sallusto, D. Lenig, R. Forster, M. Lipp, A. Lanzavecchia, Two subsets of memory T lymphocytes with distinct homing potentials and effector functions, Nature 401 (1999) 708–712.

[39] B.R. Nelson, S. Matsuhashi, F. Lefcort, Restricted neural epidermal growth factor-like like 2 (nell2) expression during muscle and neuronal differentiation, Gene Expression Patterns 2 (2002) 7–15.

[40] J. Parkin, B. Cohen, An overview of the immune system, Lancet 357 (2001) 1777–1789.

[41] Y. Tang, T.A. Glauser, D.L. Gilbert, A.D. Hershey, M.D. Privitera, D.M. Ficker, J.P. Szaflarski, F.R. Sharp, Valproic acid blood genomic expression patterns in children with epilepsy—A pilot study, Acta Neurol. Scand. 109 (2004) 159–168.

[42] R.R. Trifiletti, A.M. Packard, Immune mechanisms in pediatric neuropsychiatric disorders: Tourette's syndrome, OCD, and PANDAS, Child Adolesc. Psychiatr. Clin. North Am. 8 (1999) 767–775.

[43] S.E. Swedo, H.L. Leonard, B.B. Mittleman, A.J. Allen, J.L. Rapoport, S.P. Dow, M.E. Kanter, F. Chapman, J. Zabriskie, Identification of children with pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections by a marker associated with rheumatic fever, Am. J. Psychiatry 154 (1997) 110–112.

[44] K. Kariko, D. Weissman, F.A. Welsh, Inhibition of Toll-like receptor and cytokine signaling—A unifying theme in ischemic tolerance, J. Cereb. Blood Flow Metab. 24 (2004) 1288–1304.

[45] M. Ishikawa, J.H. Zhang, A. Nanda, D.N. Granger, Inflammatory responses to ischemia and reperfusion in the cerebral microcirculation, Front. Biosci. 9 (2004) 1339–1347.

[46] A. Chamorro, Role of inflammation in stroke and atherothrombosis, Cerebrovasc. Dis. 17 (Suppl. 3) (2004) 1–5.

[47] P.J. Lindsberg, A.J. Grau, Inflammation and infections as risk factors for ischemic stroke, Stroke 34 (2003) 2518–2532.

[48] J.A. Zeller, K. Frahm, R. Baron, R. Stingele, G. Deuschl, Platelet–leukocyte interaction and platelet activation in migraine: a link to ischemic stroke? J. Neurol. Neurosurg. Psychiatry 75 (2004) 984–987.

[49] M. Estevez, K.L. Gardner, Update on the genetics of migraine, Hum. Genet. 114 (2004) 225–235.

[50] M. Etminan, B. Takkouche, F.C. Isorna, A. Samii, Risk of ischaemic stroke in people with migraine: systematic review and meta-analysis of observational studies, BMJ 330 (2005) 63.

[51] M. Wessman, M.A. Kaunisto, M. Kallela, A. Palotie, The molecular genetics of migraine, Ann. Med. 36 (2004) 462–473.

[52] C. Iadecola, M. Alexander, Cerebral ischemia and inflammation, Curr. Opin. Neurol. 14 (2001) 89–94.

[53] M.A. Moskowitz, Neurogenic inflammation in the pathophysiology and treatment of migraine, Neurology 43 (Suppl. 3) (1993) S16–S20.

Pergamon

Life Sciences, Vol. 57, No. 17, pp. 1621-1631, 1995

0024-3205/95 $9.50 + .00

0024-3205(95)02138-8

HIGHLY-SENSITIVE IDENTIFICATION OF α-FETOPROTEIN mRNA IN CIRCULATING PERIPHERAL BLOOD OF HEPATOCELLULAR CARCINOMA PATIENTS

Naomi Funaki, Junji Tanaka, Shin-ichi Seto, Takayuki Kasamatsu, Toshimi Kaido and Masayuki Imamura.

First Department of Surgery, Faculty of Medicine,
Kyoto University,
54 Shogoinkawahara-cho, Sakyo-ku, Kyoto 606, Japan

(Received in final form August 21, 1995)

Summary

In order to capture hepatocellular carcinoma (HCC) cells in circulating peripheral blood, we made analysis to see if α-fetoprotein (AFP) mRNA exists in the peripheral blood obtained from patients with HCC and also, as a control, from hepatitis-viral-marker-positive patients without HCC and a healthy volunteer. As the number of HCC cells in peripheral blood and the quantity of AFP mRNA are expected to be very small, the analysis was performed by the reverse transcription followed by an original three-step polymerase chain reaction. By this highly-sensitive method, 5 of 7 HCC patients were positive for AFP mRNA. These 5 positive patients consisted of three with clinically apparent recurrence, one preoperative patient with tumor thrombus in the portal vein and one recurrence-free patient who developed clinically detectable recurrence three months after this analysis. Neither 4 patients with positive viral markers nor a healthy volunteer was positive. The results suggest that detection of AFP mRNA from HCC patients' peripheral blood by our highly-sensitive RT-PCR may be a practical and powerful tool to diagnose the preoperative spreading of HCC and to monitor its recurrence.

Key Words: AFP mRNA, three-step RT-PCR, hepatocellular carcinoma, hematogenous disseminations

Address correspondence to : Naomi Funaki, M.D.
First Department of Surgery, Faculty of Medicine, Kyoto University
54 Shogoinkawahara-cho, Sakyo-ku, Kyoto 606, Japan

It is ideal to perform curative liver resection for all HCC patients. But in many cases the poor hepatic functional reserve due to co-existing chronic hepatitis or liver cirrhosis limits the range of safe resection. Moreover, even if we could manage to perform curative resection, not a few patients develop recurrence. In addition, in recent years more conservative therapeutic choices such as transcatheter arterial embolization (TAE) and percutaneous ethanol infusion therapy (PEI) have been practiced. Therefore, if we could know more precisely before operation whether a patient's HCC is localized or has unhappily hematogenously disseminated, we would be able to choose more appropriate therapy for individual patients taking both the stage of HCC and hepatic functional reserve into consideration. Furthermore, the capture of circulating HCC cell(s) from a post-operative patient will enable us to predict his high possibility of recurrence, and so will tell us the timing of imaging diagnosis, or even an intensive preventive chemotherapy. By these reasons, we tried to detect HCC cells circulating in the peripheral blood as a form of AFP mRNA. AFP is a well-known oncofetal protein and its serum level is used in screening HCC (1). But secreted AFP protein does not mean the presence of "cell" in the circulation (2). In contrast, the presence of AFP mRNA in the circulation, that is, the existence of AFP-producing cell(s) in blood is thought to be highly suggestive of the existence of circulating HCC. The present study describes our successful capture of AFP mRNA from peripheral blood of pre- and post-operative HCC patients with high efficiency.

Patients and Methods

Patients All patients' profiles are listed on Table I .

RNA extraction and cDNA synthesis RNA was extracted from peripheral blood according to the acid guanidinium-phenol-chloroform (AGPC) method (3) with a slight modification. In short, 5ml of heparinized whole blood was mixed well with 5ml of a guanidine isothiocyanate-enriched solution D [5M-guanidine isothiocyanate/ 25mM-sodium citrate(pH7.0)/0.5% Sarcosyl/100mM- β -mercaptoethanol]. We increased the quantity of guanidine isothiocyanate for protection of RNA in the whole blood according to the report of Gillespie et al (4). Two ml of this sample mixure, which

TABLE I

Patient number	Sex (M/F)	Age (yr)	HCC	clinical findings	viral*1 marker	serum AFP (ng/ml)	step of detection
1.	M	65	+	R(lung)*2 LC *3	HBsAb, HCVAb	4325	second
2.	F	60	+	R(liver)	HBsAb	83	second
3.	M	66	+	R(liver) LC	HBsAb, HCVAb	18	second
4.	F	45	+	pre*4 LC	HBsAg	210	third
5.	M	56	+	R(-) LC	HBsAb	4	third
6.	M	74	+	pre LC	HCVAb	67213	-
7.	M	68	+	R(-) LC	negative	4	-
8.	M	35	-	NC*5	negative	<4	-
9.	F	54	-	CH*6	HBsAb, HCVAb	12	-
10.	F	80	-	LC	HCVAb	<4	-
11.	M	64	-	none*7	HCVAb	<4	-
12.	F	90	-	none	HBsAg	<4	-

*1 Positive marker(s) listed

*2 R indicates recurrence with the site in parentheses

*3 LC indicates liver cirrhosis

*4 'pre' indicates preoperative case

*5 NC indicates normal control

*6 CH indicates chronic hepatitis

*7 'none' in these patients means that there is no liver dysfunction at present

corresponds to 1ml of total blood, was further processed by the ordinary AGPC method. Extracted RNA was solubilized in diethyl pyrrocarbonate (DEPC)-treated water (4) and was reverse-transcribed in a 50 μl mixure consisted of 10μl of 5× buffer (Gibco BRL), 2mM dNTP (Wako Pure Chemical Industries,LTD,Japan), 10mM DTT (Gibco), 0.25μg random hexamer (Pharmacia), 5 μg bovine serum albumin (BSA)(Gibco) and 200U M-MLV reverse transcriptase (Gibco,Cat.No. 28025-013). The reverse transcription was performed at 37 ℃ for one hour.

Preparation of positive control template a high-AFP-producing human HCC cell line, HuH7 (Gift from JCRB) (5) was cultivated in Dulbecco's modified eagle's medium supplemented with 10% fetal

bovine serum. mRNA was extracted from HuH7 cells using a Quick Prep® mRNA purification kit (Pharmacia) and then reverse transcription was performed using 1 μg of mRNA according to the above-described method.

PCR primers The primers for AFP gene (6,7) detection were sense primer 1 (5'-ATTCAGACTGCTGCAGCCAA-3') and sense primer 2 (5'-GTTCCAGAACCTGTCACAAG-3') both within exon 4, sense primer 3 (5'-TGGGACCCGAACTTTCCAAG-3') within exon 6, and common anti-sense primer 4 (5'-GTGCTCATGTACATGGGCCA-3') within exon 7.

PCR protocol First step-PCR was performed using primers 1 and 4 which amplify a 476-bp fragment. The 50 μl of individual PCR mixure was composed of one half of the reverse-transcribed sample (25μl) and 5μl of 10 × PCR buffer (Perkin-Elmer-Cetus, Norwalk,CT), 50pmole of each primer in 1μl of TE(pH8.0), 1 μg of BSA, 0.2mM dNTP and DEPC-treated water. The reaction mixure was overlaid with mineral oil, heat-denatured at 93 ℃ for 3minutes and then cooled to 80℃ for addition of 2.5 units of Taq polimerase (AmpliTaq®, Perkin-Elmer-Cetus). Each cycle of amplification consisted of 1-second denature at 94℃, followed by 20-second annealing (63℃) and 30-second extension (72 ℃). After 35 cycles, the final product was extended for 10 more minutes. One-fiftieth of the first PCR product was used as the template for the second-step PCR. The second set of primers consisted of primers 2 and 4 which amplify a 384-bp fragment. These primers were added by 50pmole individually. Another constituent of the second PCR mixture was the same as the first PCR mixure except for the addition of 24μl of DEPC-treated water. The amplification program for the second PCR was the same as the first PCR. One-fiftieth of the second PCR product was used as the template for the third PCR. The third primers were 3 and 4, amplifying a 115-bp fragment. Except for the difference of primers, the third PCR mixture was the same as the second PCR mixure. The annealing step for the third PCR was performed at 65℃ for 30 seconds and 30 cycles of amplification was practiced. The rest of the amplification program for the third PCR was the same as the previous PCR. 10μl of the PCR product was subjected to electrophoresis unless noted on 2.5% agarose gels (Agarose NA,Pharmacia Biotech,Sweden) containing 20ng/ml ethidium bromide.

To test the reliability of RNA extraction, a 319-bp β-actin cDNA fragment was amplified using the 50-pmole-each primer pair reported by Fuqua et al (8). The PCR template was the residual

one half of sample cDNA. The constituent of the PCR mixture was the same as the mixture for AFP mRNA except for the difference of primers. Our PCR protocol for the detection of β-actin cDNA fragment consisted of 3-minute heat-denature at 93℃, Taq polimerase addition at 80 ℃, and then 1-second heat denature (94 ℃), followed by 30-second annealing (55℃) and 30-second extension (72 ℃) steps. After 35 cycles, the final product was extended for 10 more minutes. When the amplified band was faint, one-fiftieth of the PCR product (corresponding 1 μl) was amplified in a new PCR mixure for 30 cycles using the same primer set under the same program.

The positive control for AFP mRNA detection was the PCR performed with 0.1μg of HuH7 mRNA. The negative control for the first step was the simultaneously-performed PCR without any template. The negative control for the second- and third-step was the PCR performed with one-fiftieth (corresponding to 1 μl) of the negative control in the previous PCR.

Results

PCR amplification on HuH7 As is observed in Figure 1, the first, second or third PCR products were visible as a clear single band individually and non-specific additional bands were not observed either on positive lanes or negative control lanes.

Detection of AFP mRNA in serially diluted HuH7 cDNA To determine the sensitivity of our assay, 0.1 μg of HuH7 cDNA was serially diluted and used as the PCR template. The first PCR made a 10^{-2}-diluted sample visible (data not shown). The second PCR visualized a 10^{-6}-diluted sample (Figure 2A), and the third PCR increased the visible range up tp 10^{-8} dilution (Figure 2B).

Detection of AFP mRNA in small quantity of HuH7 cells mixed with 1ml of AFP mRNA-negative normal healthy volunteer's blood 10^{4}, 10^{3}, 10^{2}, 10, 5 or 1 HuH7 cells were mixed with 1ml of healthy volunteer's blood, which had been pre-checked as negative for AFPmRNA using our system. Total RNA extraction, cDNA synthesis and PCR amplification were performed just as was done on patients' blood. Under our system even one HuH7 cell-containing blood sample was positive for AFP mRNA and the blood sample without HuH7 cells was negative (Figure 3).

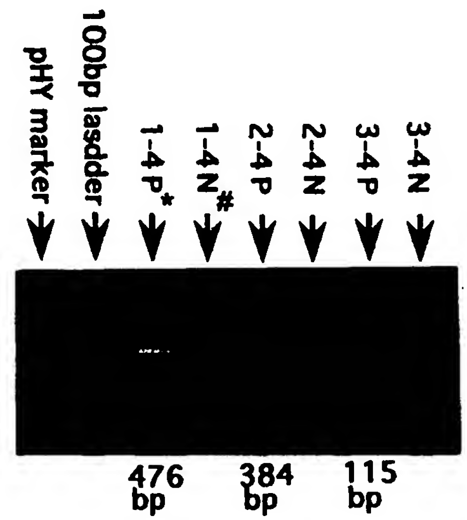


FIG.1

PCR amplification of HuH7 cDNA forms a clear single band. *P is PCR product with template and #N is that without template. Numbers 1,2,3 and 4 represent the name of primer. pHY marker indicates 4870,2016,1360,1107,926,658,489, 267 and 80bp fragments. The intermediate bright band of 100 bp-ladder indicates 600bp DNA.

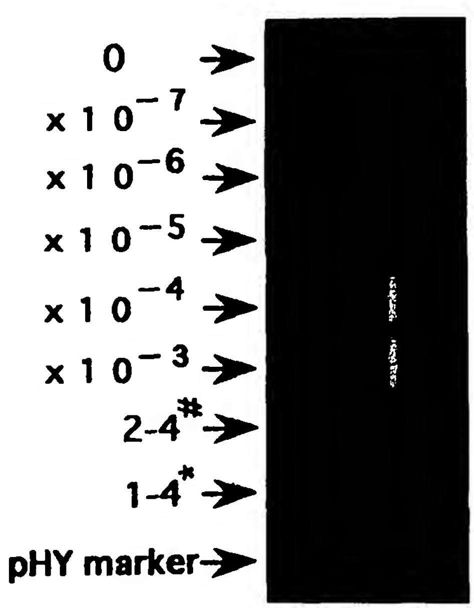


FIG.2A

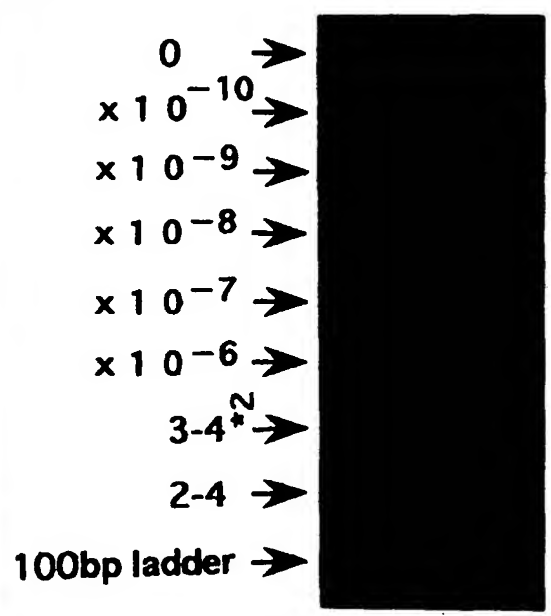


FIG.2B

PCR using serially diluted HuH7-derived cDNA to test the sensitivity. Fig.2A represents the second PCR product and Fig.2B the third PCR product. 1-4*, 2-4# and 3-4*2 are the positive control lane with indicated primer pair.

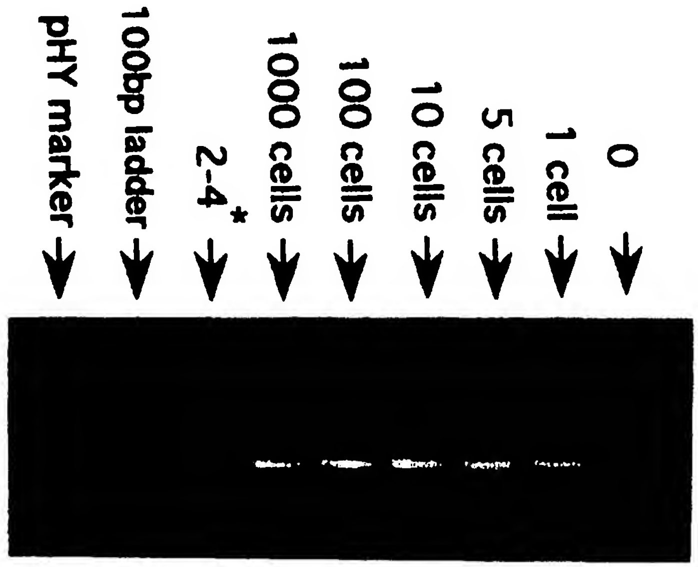


FIG.3
Even one HuH7 cell contaminated in 1ml of healthy volunteer's blood was detectable at the second PCR. 2-4* indicate the positive control product with the second primer pair. Of the total 50μ l of PCR product, we used 5 μ l for the 1000-cell and 100-cell samples, and 15μ l for the 10-cell sample, and 30μ l for the 5-cell and one-cell samples, individually.

<u>Detection of AFP mRNA in patients' samples</u> Among 7 HCC patients, 5 were positive for AFP mRNA (71%)(Figure 4). Of these 5 patients, No.1, 2 and 3 patients were already positive on the second PCR. No.1 patient had lung metastasis and No.2 and No.3 patients had multiple intrahepatic recurrence (Table 1). No.6 preoperative patient, whose serum AFP protein level was as high as 67213 ng/ml was negative for AFP mRNA. When we performed the PCR, two more patients (No.4 and 5) were found to be positive. No.4 preoperativepatient whose portal venogram showed tumor thrombosis was positivefor AFP mRNA. No.5 follow-up patient in whom no recurrence was detected at the point of blood sampling was positive for AFP mRNA, and within three months showed an evident recurrence on the echograms. No.7 follow-up patient without any clinical recurrence and negative for AFP mRNA showed no recurrence even after three months. In contrast, no AFP mRNA transcript was found in blood samples from the 4 virus-infected patients and 1 healthy volunteer (Figure 4).

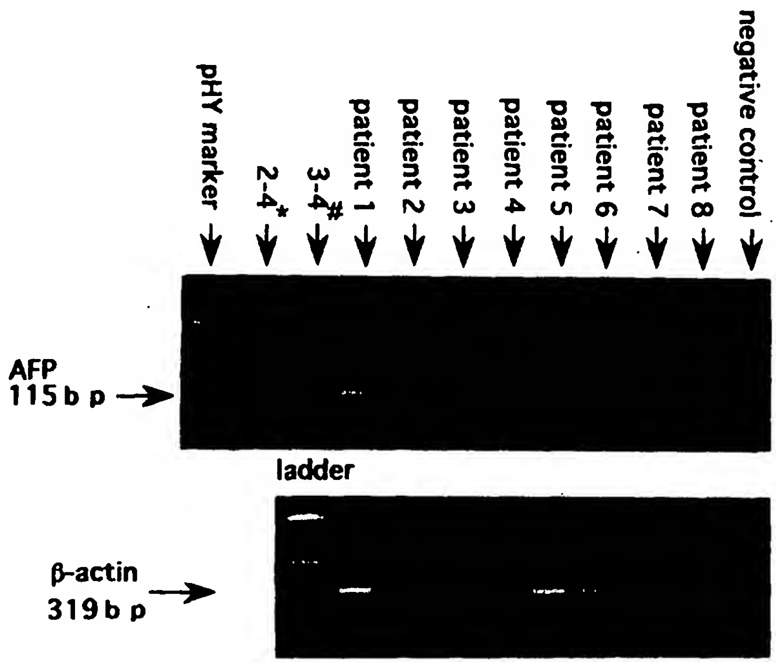


FIG.4A

The third step PCR amplification of HCC patients' sample (1-7) and a healthy volunteer's sample (8). 2-4* and 3-4# indicate the positive control for the second and third PCR, individually.

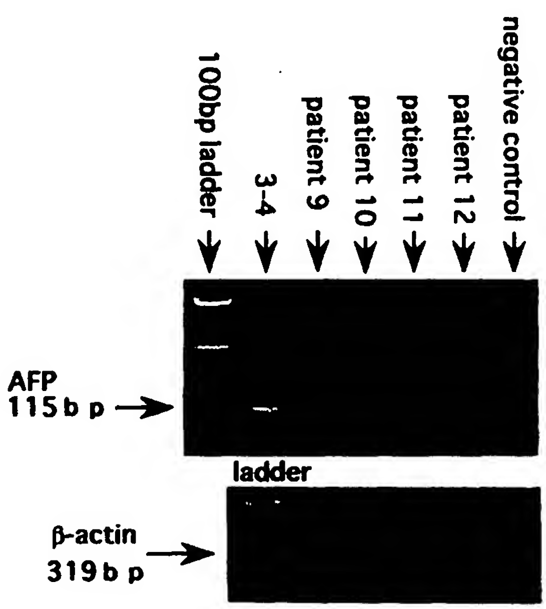


FIG.4B

PCR amplification of virus-marker-positive patients' samples

Sequencing of the final PCR product of the positive control sample and the randomly-picked up patients' samples revealed that the amplified products were identical with the expected cDNA sequence without fail.

Discussion

In the previous decade, detecting cancer-related protein secreted into blood was the only method to know the outgrowth of primary cancer or the cancer recurrence. In recent years, trials have been reported to detect cancer cell itself in the bone marrow or in the peripheral blood via RT-PCR using specific genes. To date such studies have been conducted on melanoma cells (9), breast cancer cells (10), prostate cancer cells (11), neuroblastoma cells (12) and so on. In 1994 Matsumura et al. reported a RT-PCR system detecting AFP mRNA from HCC patients' peripheral blood after collecting nucleated cell fractions (2). According to their report, the serum AFP protein level in their patients was as high as 62738$\pm$ 7031ng/ml. These HCC cells may be high-AFP-producers. And also their HCC's are exclusively large and far-advanced. For all the analysis of these advanced patients, the overall positivity within HCC patients was 52%. In order to make use of this attractive method, we hope to construct a far more sensitive system that would detect the presence of a very few number of cells and low-AFP-producing cells in the peripheral blood without consuming time for decision-making of the operative procedure and for early and direct prediction of recurrence.

First, as most HCC patients' blood is a highly infectious source of hepatitis virus, we decided to avoid the cell-fractioning procedure. In spite of having used the whole blood with the elevated RNase activity because of virus infection, we were able to obtain intact RNA enough for analysis. That is probably because of guanidine isothiocyanate-enrichment in solution D.

Second, our three-step system was able to detect the highly diluted positive control cDNA sample (Figure 2A and 2B).

Third, as we shortened each step of the PCR cycle, the blood sample obtained in the morning was processed into the final PCR product in the evening.
Using actual patients' samples, the second PCR detected AFP mRNA from 3 of a total of 7 HCC patients (43%). But the sensitivity

increased up to 71% (5 of 7) after the full three-step PCR. The serum AFP protein level of AFP mRNA-positive patients was 4325, 83, 18, 210 and 4 ng/ml, respectively. Although circulating cancer cell(s) does not necessarily adhere to and invade into patient's tissue, the presence of one patient who developed recurrence after detection of circulating AFP mRNA suggests that our system will work as a very sensitive and direct predictor of recurrence with a high probability. AFP mRNA-negative patients consisted of one preoperative patient suffering from giant HCC whose serum AFP protein level was 67213 ng/ml and one post-operative patient (serum AFP 4 ng/ml) without any recurrence even 3 months after this analysis. We also investigated patients with positive viral markers, thinking of a minor possibility of the leakage of regenerating or infected non-cancerous hepatocytes into blood (13,14). Although the number of investigated patients is four at present, neither viral-marker-positive patients nor a healthy volunteer was positive for this analysis.

When we investigated the sensitivity of our system by a mixure of HuH7 cell(s) with healthy volunteer's blood, only one cell was detected at the second PCR. The reasons for this very high sensitivity may be ① highly active AFP production by HuH7 cells (10^5 pg/24hour/10^4 cells even in FCS-free medium and the original patient's serum AFP level was more than 128000ng/ml)(6), ② high activity of HuH7 cells freshly detached from culture dish, ③ relatively low RNase activity of healthy volunteer's blood and ④ "career effect" of blood-derived RNA (compared to highly diluted cDNA alone). When we used hepatitis patient blood (negative for AFP mRNA by the blood alone) for dilution of HuH7 cells, one-cell-sample became faintly detectable after the second PCR and became clearly visible after the third PCR (data not shown). This may suggest that the four explanations are all tenable and that our three-step detection system is highly effective.

At present, no one can rule out the possibility of contamination of a few AFP-producing virus-infected hepatocytes or regenerating hepatocytes in the AFP mRNA-positive results, because there is no definite and widely-applicable marker to discriminate between cancer cells and non-cancerous hepatocytes. But non-cancerous hepatocytes are thought to be unable to live long without

anchoring, and the AFP mRNA positivity was zero among non-cancerous patients in the present analysis. Thus, our analysis seems to be able to tell a "high possibility" of hematogenous dissemination.

In conclusion, the three-step nested RT-PCR detection of AFP mRNA from peripheral whole blood seems to provide direct and practically useful information about hematogenous metastasis in both pre- and post-operative HCC patients.

References

1. K.OKUDA, K.KOTODA, H.OBATA, N.HAYASHI, T.HISAMITSU. Gastoro-enterology. 69 226-34 (1975)
2. M.MATSUMURA,Y.NIWA,N.KATO,Y.KOMATSU,S.SHIINA,T.KAWABE, T.KAWASE,H.TOYOSHIMA,M.IHORI,Y.SHIRATORI,M.OMATA. HEPATOLOGY 20 1418-25 (1994)
3. P.CHOMCZYNSKI, N.SACCHI.Anal.Biochem.162 156-9 (1987)
4. D.H.GILLESPIE,K.K.CUDDY,T.KOLBE,D.I.MARKS.Nature 367 390-391 (1994)
5. H.NAKABAYASHI, K.TAKETA, K.MIYANO, T.YAMANE, J.SATO. Cancer Research.45 6379-83 (1985)
6. T.MORINAGA,M.SAKAI,T.G.WEGMANN,T.TAMAOKI,Proc.Natl.Acad.Sci. USA.80 4604-8 (1983)
7. M.SAKAI,T.MORINAGA,Y.URANO. J.Biol.Chem. 260 5055-60 (1985)
8. S.A.FUQUA,N.F.FALETTE,W.L.McGUIRE,J.Natl.Cancer Inst.82 858-861 (1990)
9. B.SMITH, P.SELBY, J.SOUTHGATE, K.PITTMAN, C.BRADLEY, G. E.BLAIR. Lancet 338 1227-9 (1991)
10. H.D.DATTA, P.T.ADAMS, W.R.DROBYSKI, S.P.ETHIER, V.H.TERRY, M.S.ROTH. J.Clin.Oncol. 12 475-82 (1994)
11. D.P.Jr.WOOD, E.R.BANKS, S.HUMPHREYS, J.W.McROBERTS, V.M. RANGNEKAR.Cancer 74(9) 2533-40 (1994)
12. H.NAITO,N.KUZUMAKI,J.UCHINO,R.KOBAYASHI,T.SHIKANO,Y.ISHIKAWA Eur.J.Cancer 27(6) 762-5 (1991)
13. J.R.BLOOMER,T.A.WALDMANN,K.R.McINTIRE,G.KLATSKIN.JAMA 233 38-41 (1975)
14. T.TAMAOKI,N.FAUSTO, In: Stein G, Stein.J (Eds.),Recombinant DNA and cell proliferation New York:Academic Press,145-168 (1984)

H

ANNALS OF SURGERY
Vol. 226, No. 1, 43-50

Prospective Evaluation of Circulating Hepatocytes by Alpha-Fetoprotein mRNA in Humans During Liver Surgery

Antoinette Lemoine, Ph.D.,* Thierry Le Bricon, Ph.D.,* Marina Salvucci, Pharm.D.,*
Daniel Azoulay, M.D.,† Patrick Pham, Pharm.D.,* Joseph Raccuia, M.D.,‡
Henri Bismuth, M.D., FACS(Hon),† and Brigitte Debuire, Ph.D.*

From the Service de Biochmie et Biologie Moléculaire and the Centre Hépatobiliaire†—Hôpital Paul Brousse, Villejuif Cedex, France; Department of Surgery,‡ St. Vincent's Hospital, New York, New York.*

Objective
The objective of this study was to analyze the specificity of detecting liver tumor cell dissemination by alpha-fetoprotein (AFP) mRNA in peripheral blood.

Summary Background Data
Alpha-fetoprotein mRNA has been used for the detection of circulating micrometastatic tumor foci of hepatocellular carcinoma (HCC); however, the interpretation of the results has been equivocal.

Methods
Sixty-four consecutive patients with malignant HCC (n = 20), liver metastases (n = 27), or nonmalignant (n = 17) liver diseases undergoing partial or total hepatectomy and orthotopic liver transplantation were included in this prospective study from January to July 1995. Peripheral blood samples were obtained before surgery, during surgery, and after surgery (range, 6–15 months). Total mRNA was extracted from nucleated cells, and cDNA synthesis and polymerase chain reaction amplification (nested polymerase chain reaction in one tube) were performed with specific AFP primers.

Results
Preoperative AFP mRNA was detected in 20 patients (17%), of which 5 of 20 had HCC. Intraoperative assessment showed positive AFP mRNA values in a total of 34 patients (53%) with various causes, of which 8 of 20 (40%) had HCC, 17 of 27 (63%) had other malignancies, and 9 of 17 (53%) had nonmalignant diseases. Recurrent tumor in patients with HCC occurred in four cases after surgery (range, 6–15 months) and did not correlate with AFP mRNA positivity before surgery, during surgery, or after surgery.

Conclusions
Alpha-fetoprotein mRNA in peripheral blood is not a specific marker of circulating micrometastases from HCC, especially in the context of surgical treatment of HCC.

The result with both orthotopic liver transplantation (OLT) and hepatic resection with the intent to cure patients with hepatocellular carcinoma (HCC) has been dismal because of the high rate of recurrence despite thorough preoperative screening for extrahepatic disease.[1,2] This high incidence of recurrence indicates that malignant hepatocytes had entered the circulation either before or during surgery, therefore, arguing for some type of adjuvant chemotherapy when surgery is required. Detection of small numbers of circulating cells and examination of tumor markers have advanced with the refinement of molecular biology techniques. These advances have been popularized more recently with attempts to detect circulating tumor cells before dissemination is clinically apparent.[3-10] Two markers for circulating tumoral liver cells have been proposed. One involves the albumin mRNA[11-13] and, more recently, the other involves the presence of AFP mRNA in peripheral blood, which has been reported to be more specific in patients with HCC.[14-18] Particularly, these markers have been positive in patients with a high grade of HCC or with extrahepatic metastases.[15,18] However, the accuracy of this "HCC-specific" gene transcript has yet to be determined.

Equivocal interpretations from the previous studies concerning markers for circulating HCC cells prompted our group to initiate a prospective study of AFP mRNA specificity in a large group of consecutive patients undergoing either partial or total hepatectomy and OLT for various tumoral and nontumoral liver diseases. Blood samples were taken before and at two different timepoints during surgery (during the exploratory phase and after hepatectomy) to elucidate the status of AFP mRNA in the circulation of patients with malignant or nonmalignant liver disease during the course of liver surgery. We hypothesized that liver surgery would be associated with a release of hepatocytes into the bloodstream that could be detected using AFP mRNA as the target gene transcript, therefore, questioning its potential for clinical use regarding therapeutic decisions such as intraoperative chemotherapy or in the immediate postoperative period.

PATIENTS AND METHODS

Study Population

Sixty-four consecutive patients with malignant or nonmalignant liver diseases undergoing partial (n = 45) or total hepatectomy and OLT (n = 19) were included in this prospective study from January to July 1995. There were 20 patients with HCC (19 men and 1 woman), 2 with cholangiocarcinoma, 25 with liver metastases (12 men and 13 women) from colorectal carcinoma (n = 19), breast cancer (n = 3), and other types of malignancy (n = 3). Thirteen patients without liver tumor confirmed by histologic analysis had cirrhosis (11 men and 2 women), caused by alcoholism in 8 patients, chronic viral hepatitis C infection in 1, alcoholic disease associated with viral hepatitis C in 2, primary biliary cirrhosis in 1, and cirrhosis from unknown origin in 1. Four patients had neither liver tumor nor cirrhosis but two cases of amyloid neuropathy, one case of porphyria, and one case of focal nodular hyperplasia. Diagnosis of HCC was made by ultrasonography or computed tomography, serum AFP, and confirmed by final pathology results after surgical resection. Size (*i.e.*, maximal diameter of tumor), number of nodules in the liver, and total volume of the tumor were calculated using imaging and volumetric scanning techniques, including intraoperative ultrasound. Final pathology results determined the grade of HCC according to Edmonson classification, the presence of portal vein invasion, and the Child score for cirrhosis. The control group included 28 normal, healthy volunteers without liver diseases (14 men, 14 women; mean age, 30 years).

The majority of patients with HCC (15/20, 75%) received neoadjuvant chemoembolization, and serum AFP levels were normal (<20 ng/mL) at the time of the hepatectomy in 12 (60%) of 20 patients. Fifteen (56%) of the 27 patients operated on for liver metastasis from other cancers received neoadjuvant chronomodulated chemotherapy according to our institutional protocol.[19]

Alpha-Fetoprotein mRNA Assay

Peripheral blood samples were obtained before surgery and during surgery at two different intervals: the first during the exploratory phase and the second after hepatectomy was completed, using ethylenediaminetetraacetic acid as an anticoagulant. All patients surviving the early postoperative period (n = 59) were observed 6 to 15 months after hepatectomy with blood samples obtained during routine clinic examination. Sensitivity of our assay was determined with human hepatocytes isolated from cadaveric multiple organ donors.[20]

Nucleated cells were isolated from peripheral blood using tetradecyltrimethylammonium bromide as described elsewhere,[21] and total RNA was extracted from the pellet or from cryopreserved liver tissues according to our technique reported previously.[22] Special attention was given to the choice of AFP primers to avoid cross-reaction between albumin and AFP mRNA because they present a 50% homology at the mRNA level. The primers

Supported by grants from the Faculté de Médecine Paris-Sud and the Association pour la Recherche sur le Cancer (ARC).

Address reprint requests to Brigitte Debuire, Ph.D., Service de Biochimie et Biologie Moléculaire, Hôpital Paul Brousse, 14 Avenue Paul Vaillant Couturier, 94804 Villejuif Cédex, France.

Accepted for publication May 30, 1996.

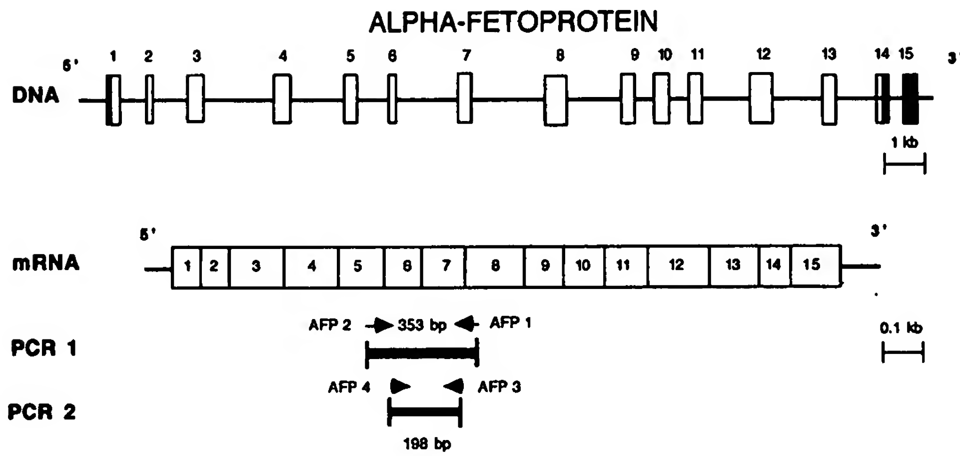


Figure 1. Representation of alpha-fetoprotein gene, alpha-fetoprotein gene transcript, and the position of primers used in reverse transcriptase-polymerase chain reaction.

were designed from the gene sequences of human serum alpha-fetoprotein.[23] To avoid false-positive results caused by DNA contamination, primers were selected in different exons of the AFP gene (Fig. 1). Sequences of primers used in the experiment were as follows. The sense primers were 5′ CAA TTC TTC TTT GGG CTG CTC GCT ATG AC 3′ (AFP 2) and 5′ ATG CAG TTG AAT GCT TCC AA 3′ (AFP 4), and the antisense primers were 5′ AGT GTC TTG TTG AGA ACA TAT GTA GGA CAT G 3′ (AFP 1) and 5′ CCA CAT CCA GGA CTA GTT TCT 3′ (AFP 3). The cDNA synthesis and polymerase chain reaction (PCR) amplification procedure (nested PCR in one tube) were performed as reported already by us.[22] Size of the amplified products of AFP mRNA was 198 base pairs.

Statistical Analysis

Statistical analysis was performed to determine if the influence of the indication for surgery (HCC *vs.* other indications), the surgical procedure itself (partial *vs.* total hepatectomy), and/or the use of neoadjuvant chemotherapy could affect detection of AFP mRNA in the circulation. The test results in the study group of HCC were correlated to the incidence of recurrence. Results are presented as mean ± standard deviation. Statistical analysis was performed with a statistical program (Statistica; StatSoft, Tulsa, OK) using the chi square test with a significance level set at 0.05.

RESULTS

Alpha-Fetoprotein mRNA Assay Sensitivity and Control Group

The sensitivity of our assay, determined in a dilution experiment (Fig. 2) using freshly isolated human hepatocytes (10^5 to 10^1) in 1 mL whole blood before RNA extraction, was approximately 1 hepatocyte for 10^5 peripheral mononuclear cells. Alpha-fetoprotein mRNA was not detected in the peripheral blood of 28 healthy subjects.

Preoperative Detection of Alpha-Fetoprotein mRNA

Before hepatectomy, AFP mRNA was detected in the blood of 11 (17%) of the 64 patients diagnosed with HCC (n = 5/20), cholangiocarcinoma (n = 1/2), colorectal carcinoma with liver metastasis (n = 4/25), and chronic active viral C hepatitis infection (n = 1/13). Patients with malignant disease and detectable AFP mRNA before hepatectomy (n = 10/47) had received chemotherapy, which was stopped 3 to 12 months before surgery (average, 6 months).

Intraoperative Detection of Alpha-Fetoprotein mRNA

During surgery, a total of 34 (53%) of 64 patients had detectable AFP mRNA at either 1 or both sampling inter-

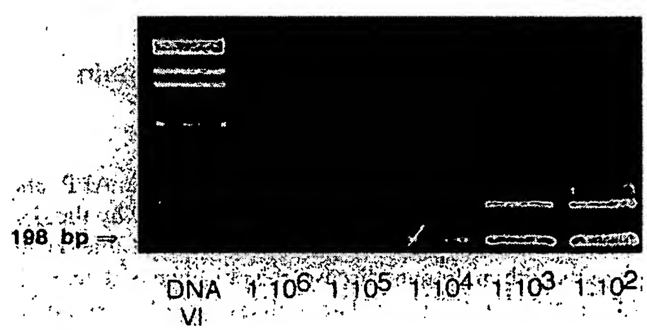


Figure 2. Sensitivity of the assay. Sensitivity of our assay was established using dilution of freshly isolated normal hepatocytes (10,[5] 10,[4] 10,[3] 10,[2] 10) in normal blood before total RNA extraction.

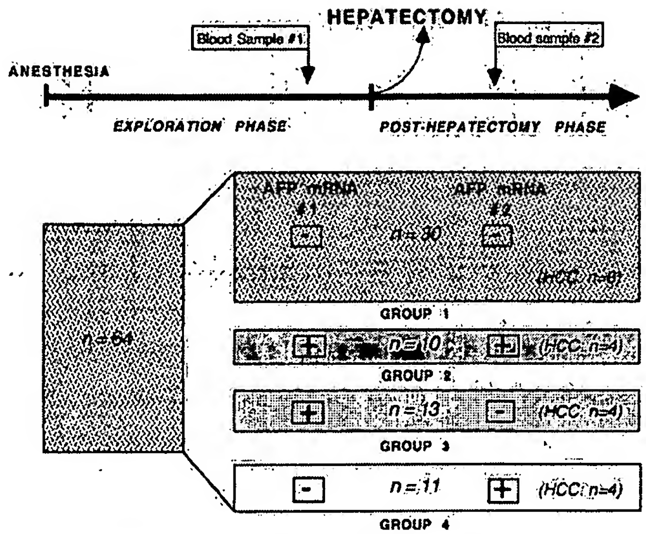


Figure 3. Schematic representation of intraoperative alpha-fetoprotein (AFP) mRNA profile in patients who are operated on. Group 1 = patients with no detectable AFP mRNA, group 2 = patients with detectable AFP mRNA at both blood samples, group 3 = patients with detectable AFP mRNA at the first blood sample only, group 4 = patients with detectable AFP mRNA at the second blood sample only.

vals. Alpha-fetoprotein mRNA was observed in 23 of the 64 patients during the exploration phase (first intraoperative blood sample), whereas 10 of these latter patients and 11 others were positive for AFP mRNA after hepatectomy (second intraoperative blood sample). Patients could be divided in four groups according to their intraoperative AFP mRNA profile (Fig. 3). In group 1, the largest (n = 30), patients had no detectable AFP mRNA during liver surgery, including 8 patients who received partial hepatectomy for HCC. In group 2 (n = 10, including 4 HCC), patients had detectable AFP mRNA at both the first and the second operative timepoints. In group 3 (n = 13, including 4 patients treated for HCC), patient results were positive at the first blood sampling and then negative in the second. Conversely, in group 4 (n = 11, including 4 patients treated for HCC), patient results were negative at the first blood sampling and positive after hepatectomy.

Correlations Between Alpha-Fetoprotein mRNA Test Results and Clinical Characteristics

The presence of detectable intraoperative AFP and mRNA was not influenced significantly either by the disease or by the type of surgical procedure (Table 1). In patients with malignant disease, the presence of detectable AFP mRNA did not correlate with the use of neoadjuvant chemotherapy (Table 1). The 20 patients with HCC included 4 patients receiving OLT and 16 having partial hepatectomy (Table 2). Forty percent of patients with HCC had more than one tumor foci, and the majority (n = 17, 85%) were encapsulated tumors. None of the tumors were of the fibrolamellar variety. Hepatocellular carcinoma was impossible to grade in 60% of patients because of tumor necrosis, whereas in the remaining patients, only three were classified as grade III or IV. Vascular invasion was present in 50% of the patients examined. No AFP mRNA was detected in 40% (n = 8) of patients who were operated for HCC (Table 2, Fig. 4). During surgery, in 40% of cases, AFP mRNA was detected in one of the two intraoperative samples, and four patients had detectable AFP mRNA at both sampling points. There was no evident relation between the presence of AFP mRNA in the blood during surgery and the serum level of the tumor marker AFP, the characteristics of the tumor (in particular the size and number of tumor foci), and the presence of vascular invasion.

Correlations Between Alpha-Fetoprotein mRNA Test Results and Clinical Outcome

Complete follow-up with peripheral blood sampling was performed 6 to 15 months after surgery. Eighteen (28%) of 64 patients had detectable AFP mRNA (1/17 nontumor causes, 7/27 secondary metastases, and 10/20 HCC) during the course of follow-up. The patient who had a signal for AFP mRNA without hepatic tumor had active chronic viral C hepatitis, whereas in the group of patients with metastatic tumor, only two of the four who had positive results before surgery remained with positive results 8 and 11 months after surgery, respectively. In the group of patients with HCC, three died in the immediate postoperative period, two from terminal liver failure and one from primary nonfunction after transplantation. These were three patients without detectable AFP mRNA during surgery (patient 20; Table 2) or only one positive test result during surgery (patients 16, 17; Table 2). The follow-up available for the 17 surviving patients ranges from 6 to 14 months (median, 9 months). Ten patients with HCC had positive results and seven had negative results for AFP mRNA signal after surgery. Among the five patients who had a signal for AFP mRNA before surgery, only one had a negative result 9 months after surgery (patient 1, Table 2). There were three patients without detectable AFP mRNA during surgery that remained with negative results (patients 1, 2, 11), whereas the results of three others became positive during surgery (patients 3, 12, 14). Two patients in the latter group had recurrent viral C hepatitis. In the group of patients that had detectable AFP mRNA during surgery, the results of seven remained positive (patients 4, 5, 6, 7, 10, 13, 18), whereas the results of four others became negative by subsequent testing (patients 8, 9, 15, 19). Finally, four patients[8-10,13]

Table 1. INFLUENCE OF INDICATION, TYPE OF SURGERY, AND PREOPERATIVE CHEMOTHERAPY ON INTRAOPERATIVE ALPHA-FETOPROTEIN mRNA IN PERIPHERAL BLOOD*

	Number of Patients (n = 64)	Alpha-Fetoprotein mRNA		
		Negative (n = 30)	Positive (n = 34)	
Indication				
Hepatocellular carcinoma	20	8 (40)	12 (60)	NS
Liver metastasis of other cancers	27	11 (41)	16 (59)	
Nonmalignant liver diseases	17	11 (65)	6 (35)	
Surgery				
Total hepatectomy and OLT	19	14 (74)	5 (26)	NS
Partial hepatectomy	45	17 (38)	28 (62)	
Chemotherapy				
Yes	28	10 (36)	18 (64)	NS
No	20	7 (35)	13 (65)	

OLT = orthotopic liver transplantation; NS = not significant.

* Patients were considered negative if no AFP mRNA was detected intraoperatively, positive if AFP and mRNA was detected in at least one of the two intraoperative samples. Chemotherapy was performed preoperatively in 28 of 47 patients with malignant liver disease. Statistical analysis was performed using the chi square test (significance set at $p < 0.05$). Values are number (%).

Table 2. HISTOLOGIC PARAMETERS OF PATIENTS OPERATED FOR HEPATOCELLULAR CARCINOMA

Patient Number	Preoperative AFP (ng/mL)	Chemoembolization	Number (size, cm) of Tumors*	Capsule	Grade†	Liver	Vascular Invasion	AFP mRNA‡				Recurrence
								Pre	1	2	Post	
1	<20	Yes	>3 (15)	No	II	NC	Yes	+	–	–	–	No
2	<20	No	1 (20)	Yes	I	NC	Yes	–	–	–	–	No
3	<20	Yes	2 (9)	Yes	II	NC	Yes	–	–	–	+	No
4	<20	Yes	1 (5)	Yes	N	C	Yes	+	+	+	+	No
5	215	Yes	1 (5)	Yes	N	NC	No	–	+	–	+	No
6	<20	Yes	3 (5)	Yes	N	NC	No	+	+	–	+	No
7	<20	No	1 (1.5)	Yes	N	C	No	+	–	+	+	No
8	10,350	Yes	1 (6)	Yes	III or IV	C	No	–	–	+	–	Yes
9	694	Yes	1 (4.5)	Yes	N	C	No	–	–	+	–	Yes
10	<20	Yes	2 (4)	Ruptured	III	NC	Yes	–	+	–	+	Yes
11	99	Yes	>3 (14)	Yes	II	NC	Yes	–	–	–	–	No
12	<20	Yes	2 (4)	Yes	N	C	No	–	–	–	+	No
13	2056	Yes	2 (7)	Yes	III	NC	No	–	+	+	+	Yes
14	593	Yes	1 (15)	Yes	N	NC	Yes	–	–	–	+	No
15	<20	Yes	2 (3)	No	N	C	No	–	–	+	–	No
16	40	No	1 (3)	No	II	NC	Yes	–	+	–	ND	Died
17	<20	No	1 (2)	Yes	N	C	No	–	–	–	ND	Died
18	<20	Yes	1 (6)	Yes	N	C	Yes	+	+	+	+	No
19	<20	Yes	1 (8)	Yes	N	NC	Yes	–	+	+	–	No
20	<20	Yes	1 (10)	Yes	N	C	No	–	–	–	ND	Died

AFP = Alpha-fetoprotein; N = necrosis; NC = noncirrhotic; C = cirrhotic; ND = not done.

* For multiple tumors, the size of the tumor indicated in the parentheses is the size of the largest one.

† Grade of the tumor was determined according to Edmonson. None of the patients studied had metastases to lymph nodes or distant organs.

‡ AFP mRNA was detected during partial hepatectomy or orthotopic liver transplantation (patients 7, 10, 11, and 12) at two time points (exploration phase, 1; after hepatectomy, 2) and 6–15 months later.

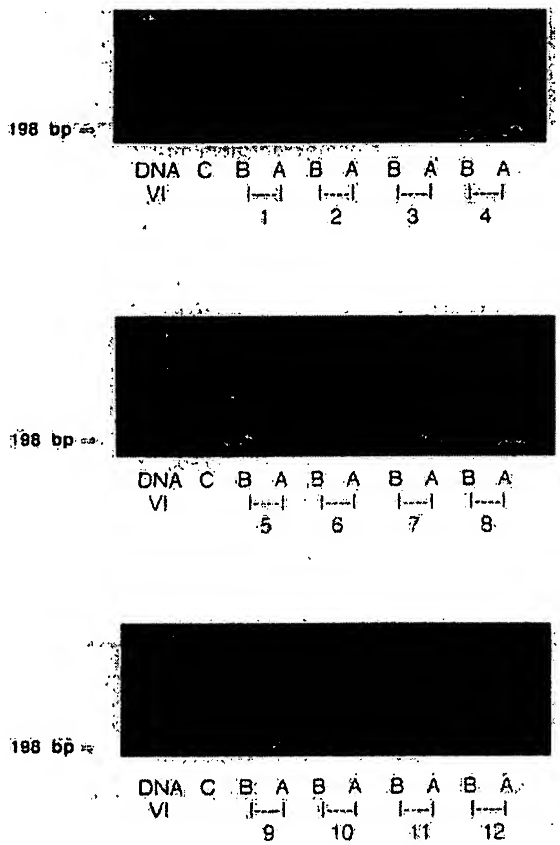


Figure 4. Electrophoresis pattern of 12 randomly chosen patients with hepatocellular carcinoma in our series of 20 cases. (A) After partial or total hepatectomy (second intraoperative blood sample) and (B) before partial or total hepatectomy (first intraoperative blood sample). Detailed characteristics of these patients are provided in Table 2. DNA VI = size marker, (C) negative control sample. Larger bands correspond to residual amplification products of the first polymerase chain reaction round.

had detectable recurrence by abdominal ultrasound, thoracoabdominal computed tomographic scan, bone scintiscan, or serum AFP levels. Of these four patients with recurrence, all had negative preoperative AFP mRNA values, but all had positive values shown during the intraoperative examination. In the immediate postoperative period, the test result was positive in only two of the patients with recurrent disease. There was no correlation between the incidence of recurrence and the test results before, during, and after surgery.

DISCUSSION

The results of this prospective study to evaluate AFP mRNA in peripheral blood lead us to conclude that this is not specific for circulating tumor foci. The use of this test, especially in the context of surgical treatment of HCC, cannot yet be recommended despite the suggestion of others in previous reports that AFP mRNA or albumin mRNA could be used clinically as a specific indicator of hematogenous dissemination of HCC cells.[11–18] We, as others,[14,15] examined albumin mRNA in the peripheral blood by PCR; however, its presence was shown in all control samples tested (n = 28, data not shown), whereas none of the control samples using AFP mRNA had tested positive. These results give credence to the concept of "illegitimate" transcription of the albumin gene in mononuclear cells.[14,15,24] Consequently, the detection of AFP gene transcripts seemed to be a valid method to identify abnormal liver cells in the circulation and, therefore, the possibility to visualize circulating HCC micrometastatic foci. None of our control patients had a positive signal for AFP mRNA, as also reported previously.[15]

Before surgery, we found a similar rate of detection in patients with HCC (25%) than in patients with other malignant liver diseases (20%). This is the first report documenting an association between detectable AFP mRNA in the circulation and liver metastases of other cancers. In the series of Komeda et al.,[18] none of the eight patients with metastatic liver cancer exhibited AFP mRNA in the blood, although another patient without cancer and chronic active viral C hepatitis had a signal for AFP mRNA before surgery. The presence of AFP mRNA already has been reported in cirrhosis (0%–15%) as well as acute and chronic hepatitis (0%–75%).[11,15–17] However, if only the preoperative test results of the nonmalignant patients (17 nonmalignant liver disease and 28 healthy control subjects) are considered, then only 1 of 45 was positive for AFP mRNA. The percentage of detectable AFP mRNA before surgery in patients with HCC (25%) was lower than that from previous results reported by others (36%–52%).[15,18] These differences cannot be explained by a lower sensitivity of our technique because a reverse transcription followed by nested PCR assay in one tube[25,26] is considered the most sensitive available (theoretically one abnormal cell for 10^6) with limited cross-contamination and is perfectly adapted to clinical laboratory use. In addition, when compared to methods involving preliminary isolation of cells,[12,15,17] our processing of blood samples is rapid, easy, and inexpensive. Furthermore, in our experience, techniques using Ficoll (Pharmacia, Uppsala, Sweden) cell extraction or dextran-based technique described by Matsumura et al.[16] obtained approximately ten times lower sensitivity than with tetradecyltrimethylammonium bromide (data not shown). Differences in AFP mRNA results in patients with HCC are more likely explained by the characteristics of patients examined, because in our report, only 3 patients (15%) had HCC grade III or IV, 12 other patients showed total tumor necrosis, thus rendering it impossible to grade, and no patient had known extrahepatic disease. This suggests that in these patients, few or no intact tumor cells would be circulating in the bloodstream. The fact that most patients

(75%) had received neoadjuvant arterial chemotherapy before surgery could explain why AFP serum levels were normal (<20 ng/mL) in many patients (65%). Although all of these factors individually might influence the potential for dissemination and subsequent detection of AFP mRNA, this has yet to be elucidated completely. In the series by Komeda et al.,[18] the presence of AFP mRNA in peripheral blood clearly was related to the stage of the disease (10% in stages I and II, 30% in stage III, and 77% in stage IV). However, the use of AFP mRNA as a marker of tumor dissemination would be useful if the tumor is at a stage when it is still considered curable, such as with stage I and II disease when AFP levels still are presumably at low levels in the group of patients examined in our series. The use of AFP mRNA in patients with late-stage (stage IV) disease seems elaborate considering that even if specific for metastases, this information most likely would be ascertained by more conventional clinical imaging methods. The subset of patients who theoretically would benefit would be at clinical stage I or II disease with positive AFP mRNA results, which then would alter the clinical therapeutic decision in the form of treatment rendered.

In our group of 64 consecutive patients undergoing either partial or total hepatectomy, detection of AFP mRNA in peripheral blood intraoperatively was possible in 53%, regardless of the disease. Detectable AFP mRNA could not be explained by the surgical indication (60% for HCC *vs.* 59% for liver metastases), and no significant differences were observed between partial or total hepatectomy (62% *vs.* 26%). In addition, the use of neoadjuvant chemotherapy (64% *vs.* 65%) and total or partial vascular exclusion of the liver during hepatectomy (data not shown) did not affect significantly the incidence of detecting AFP mRNA in peripheral blood. Our results suggest that liver manipulation clearly leads to hematogenous spread of cells from liver origin, which can be detected by AFP mRNA. The release of cancer cells has been shown during tumor resection by immunocytochemical techniques and PCR in breast cancer,[7] colorectal cancer,[27] and primary renal cancer.[28] Similarly, chemotherapy has been shown to induce a release of circulating AFP mRNA-positive cells in the circulation in small groups of patients with HCC.[18] Alpha-fetoprotein gene transcripts are found not only in HCC cells, but also in normal liver cells[11]; therefore, the malignant origin of the detected cells cannot be established or ruled out. Surprisingly, AFP mRNA signal fluctuates rapidly during the course of liver surgery. For example, patients in group 3 have a release of hepatocytes at the time of the first blood sample but not at the second endpoint, theoretically the most traumatic period. These results concur with other experimental[25,26] and clinical data[27,28] suggesting that release of abnormal cells in the circulation, either spontaneously or secondary to surgical manipulation, is an intermittent and transient phenomenon. As a result, detection of AFP mRNA might instead be dependent on the timing of the peripheral blood sampling, which further indicates the high likelihood of a "sampling error" during surgery.

During follow-up, 17 (27%) of 64 had positive AFP mRNA signals, of which 3 had chronic active viral C hepatitis. Follow-up results of patients operated on for breast cancer[7] or after chemotherapy for HCC[18] have shown that previously positive samples failed to show target gene expression 24 hours and 7 days after treatments, respectively. Analysis of HCC in our series shows ten with positive signals, whereas three had negative signals intraoperatively. Subsequently, only four patients with HCC had recurrent disease diagnosed; however, only two of these patients tested positive for AFP mRNA. The sensitivity and specificity of this test for clinical decisions, especially regarding therapy, were low (50% and 36%, respectively) with a predictive value of only 18%. This is contrary to previous studies because in only one report, disease recurrence occurred in three patients when albumin mRNA was measured in the peripheral blood of nine patients with HCC (stages II and IV) after OLT.[12] It cannot be overemphasized that reports of illegitimate transcription have been well documented with the use of albumin mRNA[14,15,24] since the test was described initially.[11,12] These results indicate that the malignant origin of detected circulating cells cannot be established by AFP mRNA that fluctuates over time, thus could represent a "sampling error." Therefore, the prognostic value of intraoperative circulating cancer cells shows both low sensibility and specificity.

The current study does reflect the inaccuracy of using the AFP mRNA as a biologic marker for detecting circulating tumor micrometastases in HCC and, therefore, the AFP gene cannot be considered an "HCC-specific" gene. Thus, suggesting different treatment options for patients with intraoperative detection of AFP mRNA cannot be advocated, and further studies are necessary to isolate certain genetic markers, which are specific for micrometastatic foci of HCC. Rather than the use of "tissue-specific" genes, potential approaches for the detection of micrometastases include the identification of "metastatic" genes, which are expressed only in metastatic cells such as the one coding for CD44, a glycosylated cell surface adhesion molecule, or the detection of an "HCC-specific" form of serum AFP. Until an accurate test is devised, it would be imprudent to attempt clinical application of AFP mRNA in the detection of micrometastatic foci.

Acknowledgments

The authors thank the staffs of the anesthesy unit and of the operating room.

References

1. Farmer DG, Rosove MH, Shaked A, Busuttil RW. Current treatment modalities for hepatocellular carcinoma. Ann Surg 1994; 219:236–247.
2. Okuda K. Hepatocellular carcinoma: recent progress. Hepatology 1992; 15:948–963.
3. Israeli RS, Miller WH, Su SL, et al. Sensitive nested reverse transcription polymerase chain reaction detection of circulating prostatic tumor cells: comparison of prostate-specific membrane antigen and prostate-specific antigen-based assays. Cancer Res 1994; 54:6306–6310.
4. Moreno JG, Groce CM, Fischer R, et al. Detection of hematogenous micrometastasis in patients with prostate cancer. Cancer Res 1992; 52:6110–6112.
5. Leather AJM, Gallegos NC, Kocjan G, et al. Detection and numeration of circulating tumor cells in colorectal cancer. Br J Surg 1993; 80:777–780.
6. Glaves D, Huben RP, Weis L. Haematogenous dissemination of cells from human renal adenocarcinomas. Br J Cancer 1988; 57:32–35.
7. Brown DC, Purushotham AD, Birnie GD, Georges WD. Detection of intraoperative tumor cell dissemination in patients with breast cancer by use of reverse transcription and polymerase chain reaction. Surgery 1995; 117:96–101.
8. Smith B, Selby P, Southgate J, et al. Detection of melanoma cells in peripheral blood by means of polymerase chain reaction. Lancet 1991; 338:1227–1229.
9. Naito H, Kuzumaki N, Uchino J, et al. Detection of tyrosine hydroxylase mRNA and minimal neuroblastoma cells by the reverse transcription-polymerase chain reaction. Eur J Cancer 1991; 27:762–765.
10. Carr BI, Kar S. An assay for hepatoma micrometastasis: albumin gene expression in peripheral blood from patients with advanced hepatocellular carcinoma (HCC). Hepatology 1991; 14:108A.
11. Hillaire S, Barbu V, Boucher E, et al. Albumin messenger RNA as a marker of circulating hepatocytes in hepatocellular carcinoma. Gastroenterology 1994; 106:239–242.
12. Kar S, Carr B. Detection of liver cells in peripheral blood of patients with advanced-stage hepatocellular carcinoma. Hepatology 1995; 21:403–407.
13. Carr BI. An assay for micrometastatic hepatocellular carcinoma. Gastroenterology 1994; 106:264–267.
14. Chou HC, Sheu JC, Huang GT, et al. Albumin messenger RNA is not specific for circulating hepatoma cells. Gastroenterology 1994; 107:630–631.
15. Matsumara M, Niwa Y, Kato N, et al. Detection of alpha-fetoprotein mRNA, an indicator of hematogenous spreading hepatocellular carcinoma, in the circulation: a possible predictor of metastatic hepatocellular carcinoma. Hepatology 1994; 20:1418–1425.
16. Matsumura M, Niwa Y, Hikiba Y, et al. Sensitive assay for detection of hepatocellular carcinoma associated gene transcription (alpha-fetoprotein mRNA) in blood. Biochem Biophys Res Comm 1995; 207:813–818.
17. Nambu S, Nishimori H, Saeki M, et al. Alpha-fetoprotein messenger RNA in peripheral blood as a marker of circulating hepatocellular carcinoma cells. Int Hep Comm 1995; 3:217–221.
18. Komeda T, Fukuda Y, Sando T, et al. Sensitive detection of circulating hepatocellular carcinoma cells in peripheral venous blood. Cancer 1995; 75:2214–2219.
19. Levi F, Zidani R, Vannetzel JM, et al. Chronomodulated versus fixed-infusion-rate delivery of ambulatory chemotherapy with oxaliplatin; fluorouracil, and folinic acid (Leucovorin) in patients with colorectal cancer metastases: a randomized multi-institutional trial. J Natl Cancer Inst 1994; 26:1608–1616.
20. Pichard L, Fabre I, Daujat M, et al. Effect of corticosteroids on the expression of cytochromes P450 and on cyclosporin A oxidase activity in primary cultures of human hepatocytes. Mol Pharmacol 1992; 41:1047–1055.
21. MacFarlane DE, Dahle CE. Isolating RNA from whole blood. The dawn of RNA-based diagnosis. Nature 1993; 362:186–188.
22. Frenoy N, Chabli A, Sol O, et al. Application of a new protocol for nested PCR to the detection of minimal residual bcr/abl transcripts. Leukemia 1994; 8:1411–1414.
23. Gibbs PEM, Zielinski R, Boyd C, Dugaiscyk A. Structure, polymorphism, and novel repeated DNA elements revealed by a complete sequence of the human alpha-fetoprotein gene. Biochemistry 1987; 26:1332–1343.
24. Chelly J, Concordet JP, Kaplan JC, Kahn A. Illegitimate transcription: transcription of any gene in any cell type. Proc Natl Acad Sci U S A 1989; 86:2617–2621.
25. Mayew E, Glaves D. Quantification of the tumorigenic dissemination of arrested cancer cells. Br J Cancer 1984; 50:159–166.
26. Fidler IJ. Metastasis: quantitative analysis of distribution and fate of tumor cell emboli labeled with ^{125}I-5-iodo-2-deoxyurinide. J Natl Cancer Inst 1970; 45:773–782.
27. Leather AJM, Gallegos NC, Kocjan G, et al. Detection and numeration of circulating tumor cells in colorectal cancer. Br J Surg 1993; 80:777–780.
28. Glaves D, Huben RP, Weis L. Haematogenous dissemination of cells from human renal adenocarcinomas. Br J Cancer 1988; 57:32–35.

Detection of α-Fetoprotein mRNA, an Indicator of Hematogenous Spreading Hepatocellular Carcinoma, in the Circulation: A Possible Predictor of Metastatic Hepatocellular Carcinoma

MASAYUKI MATSUMURA,[1] YASURO NIWA,[1] NAOYA KATO,[1] YUTAKA KOMATSU,[1] SHUICHIRO SHIINA,[1] TAKAO KAWABE,[1] TAKEO KAWASE,[1] HIROSHI TOYOSHIMA,[2] MASASHI IHORI,[2] YASUSHI SHIRATORI[1] AND MASAO OMATA[1]

[1]*Second Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, and* [2]*Department of Gastroenterology and Hepatology, Japanese Red Cross Medical Center, 4-1-22 Hiroo, Shibuya-ku, Tokyo 150, Japan*

We attempted to detect circulating hepatocellular carcinoma by demonstrating hepatocyte-associated mRNA in the nuclear cell component of peripheral blood using nested reverse transcription–polymerase chain reaction because of the extremely small number of tumor cells in the circulation. Albumin mRNA was demonstrated not only in the liver tissue (hepatocytes) and HepG2 cells but also in nuclear cells of the blood from normal healthy volunteers (neutrophils and lymphocytes) by reverse transcription–polymerase chain reaction. In contrast, α-fetoprotein mRNA was demonstrated in the liver tissue, as well as in HepG2 cells, but not in peripheral blood of normal healthy volunteers, indicating the possibility of using α-fetoprotein mRNA for detection of benign and malignant hepatocytes among the population of neutrophils and lymphocytes. α-Fetoprotein mRNA in peripheral blood was detected in 17 of 33 cases of hepatocellular carcinoma (52%), 2 of 13 cases of cirrhosis (15%) and 2 of 17 cases of chronic hepatitis (12%). α-Fetoprotein mRNA was not demonstrated in 26 cases of normal healthy volunteers (0%). Among the patients with hepatocellular carcinoma, total volume of tumor tissue, maximum size of tumor and serum α-fetoprotein level were markedly increased in the patients with α-fetoprotein mRNA in blood. In addition, α-fetoprotein mRNA was detected in the blood of all 6 patients showing metastasis at extrahepatic organs (100%), in contrast to 11 of 27 cases without metastasis (41%). From these results, we conclude that the presence of α-fetoprotein mRNA in peripheral blood may be an indicator of circulating malignant or benign hepatocytes, which might predict hematogenous spreading metastasis of tumor cells in patients with hepatocellular carcinoma. (HEPATOLOGY 1994;20:1418-1425.)

HCC often develops in patients with chronic hepatitis and cirrhosis in association with HCV or HBV infection. The number of patients with HCC in association with HCV infection has increased recently, and the incidence of HCC among patients with cirrhosis is more than 6%/yr (1).

HCC is detected with several imaging techniques such as ultrasonography, abdominal angiography and computed tomography. HCC patients undergo medical and surgical treatments such as resection of the liver lobule (2), transarterial embolization (3) and percutaneous ethanol injection therapy (4). However, intrahepatic or extrahepatic metastasis to lung, bone and adrenal glands is frequently found; this is an indicator of poor prognosis in these patients (5). In recent years, liver transplantation has frequently been performed in patients with cirrhosis at an advanced stage, but these patients sometimes have HCC. However, extrahepatic metastasis of HCC reduces the beneficial effects of liver transplantation (6). Therefore early detection of micrometastasis of HCC could be a tool useful in selection of patients with HCC for liver transplantation. However, it is very difficult to demonstrate circulating malignant cells in blood morphologically; the number of malignant cells in the circulation is extremely small.

Recent studies have reported that tumor-specific genes are used to detect tumor cells in the circulation (7, 8). In relation to the biology of hepatocytes and HCC, these cells are known to produce albumin, AFP or both (9, 10). Therefore the detection of these hepatocyte-associated mRNAs in nuclear cell components of pe-

Received November 18, 1993; accepted June 1, 1994.

Other abbreviations used in the text: AFP, α-fetoprotein; DEPC, diethylpyrocarbonate; EDTA, ethylenediaminetetraacetate; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; RT-PCR, reverse transcription–polymerase chain reaction.

This work was supported by grants from Ministry of Education, Culture and Sciences of Japan.

Address reprint requests to: Masayuki Matsumura, M.D., Second Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan.

0270-9139/94 $3.00 + 0 31/1/59373

TABLE 1. Patient profiles

Characteristics	HCC	Liver cirrhosis	Chronic hepatitis	Control
No. of patients	33	13	17	26
Age (yr)[a]	63 ± 9 (33-84)	65 ± 8 (45-75)	47 ± 12 (27-61)	57 ± 16 (33-83)
Sex (M/F)	26:7	7:6	15:2	18:8
Viral serological markers				
HBs Ag positive	6	2	0	0
HCV Ab positive	24	7	17	–
HBs Ag, HCV antibody negative	3	4	0	26
Child classification				
A	3	6	–	–
B	18	4	–	–
C	12	3	–	–

[a]Data expressed as mean ± S.D. (range).

ripheral blood may indicate the presence of circulating malignant or benign hepatocytes.

Human albumin (11) and AFP (12) genes, consisting of 14 exons tandemly located at the fourth chromosome, are 19 and 17 kb long, respectively. The expression of these genes is regulated by variable mechanisms such as colloid osmotic pressure through the modulation of the promoter activities of these genes (13). AFP, an oncofetal protein, is synthesized mainly in hepatocytes of fetal liver and yolk sac, and AFP production by hepatocytes is rapidly reduced from the time of birth in inverse proportion to an increased synthesis of albumin (10, 14). AFP mRNA has lately been detected in tumor tissue of HCC but not in nontumorous tissue, and its gene expression is reported to be modulated by hypomethylation of the AFP gene (15). Hitherto, increased serum AFP concentration, a tumor marker in patients with HCC, is used in screening for HCC (16), but it cannot predict metastasis of HCC. The presence of circulating HCC cells, which may be released from tumor foci into the circulation, could be an indicator of hematogenous spread of tumor cells leading to extrahepatic metastasis of HCC.

In this study, in an attempt to demonstrate the possibility of hematogenous metastasis of HCC, we tried to detect circulating HCC, which might be released from tumor foci into the circulation, by detecting the hepatocyte-associated mRNA (albumin or AFP mRNA) in the nuclear cell component of peripheral blood using nested RT-PCR.

PATIENTS AND METHODS

Patients. Thirty-three patients with HCC (26 men and 7 women), 13 cases of cirrhosis (7 men and 6 women) and 17 cases of chronic hepatitis (15 men and 2 women), admitted at our hospital between September 1992 and June 1993, were examined. As controls, 26 age-matched normal, healthy volunteers without liver diseases (18 men and 8 women) were selected. HBsAg and HCV antibody in serum were assayed with the Reversecell kit (Yamanouchi Pharmaceutical Company Ltd., Tokyo, Japan) and with the HCV PHA Dainabot kit (Dainabot, Tokyo, Japan), respectively. As indicated in Table 1, HCV antibody was found in more than 50% of the patients with chronic hepatitis, cirrhosis and HCC, but HBsAg was detected in fewer than 20%. Diagnosis of HCC was made by ultrasonography or computed tomography and confirmed by histology of tumor tissue. Size (maximum diameter of tumor), the number of tumor foci in the liver and total volume of tumor tissue were calculated with several imaging techniques. Diagnosis of cirrhosis and chronic hepatitis was made on the basis of liver histology, as well as clinical data. Blood was collected from a peripheral vein in a disposable syringe containing 0.1% EDTA before transarterial embolization, percutaneous ethanol injection therapy or both.

In addition, blood was collected from the hepatic vein, aorta and inferior vena cava during cardiac catheterization in 15 patients with ischemic heart diseases. Among these patients, one had cirrhosis, one had chronic hepatitis in association with HCV infection and two had fatty liver (slightly increased AST/ALT concentrations in serum).

Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in *a priori* approval by the institution's human research review committee.

Preparation of Nuclear Cells from Peripheral Blood. Five milliliters of blood was mixed with 1 ml of 5% dextran–saline solution and left to set for 30 min at room temperature to yield erythrocyte sediment (17). Supernatant was collected and centrifuged at 500 *g* for 25 min. The cells were then mixed with autoclaved, distilled water for lysis of residual erythrocytes. We then restored isotonicity by adding the same volume of 1.8% NaCl solution after 25 sec. After centrifugation at 350 *g*, the cells were immediately frozen with liquid nitrogen and stored at −80° C until use.

Liver Specimens and Cell Line of Hepatocytes. Normal liver tissue was obtained from autopsied liver. HepG2 cell line (a gift from Dr. Makoto Noda in Riken Cell Bank; RCB459) was maintained in Dulbecco's minimal essential medium (Gibco-BRL, Grand Island, NY) containing 10% fetal calf serum (Gibco-BRL).

Extraction of RNA. RNA was extracted from blood nuclear cells, liver tissue and HepG2 cells by use of RNAzol B (Biotecx Laboratories, Houston, TX) according to the manufacturer's protocol. RNA was then dissolved in DEPC-treated water and stored at −80° C until use.

Synthesis of cDNA. One microgram of RNA, which was heated at 95° C for 5 min and cooled rapidly on ice, was diluted at a volume of 10 μl containing 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol, 0.25 μg hexamer random primer (Takara Biochemicals, Kyoto, Japan), 18 units of RNAse inhibitor (Takara Biochemicals) and 200 units of M-MLV reverse transcriptase (Gibco-BRL). cDNA was synthesized by means of incubation at 42° C for 60 min. It

TABLE 2. Detection of AFP mRNA in nuclear cells of peripheral blood

Group	No.	AFP mRNA in blood		
		Positive	Negative	% Positive
HCC				
TOTAL	33	17	16	52%[a]
HBs Ag positive	6	4	2	
HCV Ab positive	24	11	13	
HBs Ag, HCV antibody negative	3	2	1	
Cirrhosis				
TOTAL	13	2	11	15%
HBs Ag positive	2	1	1	
HCV Ab positive	7	0	7	
HBs Ag, HCV antibody negative	4	1	3	
Chronic hepatitis				
TOTAL	17	2	15	12%
HBs Ag positive	0	0	0	
HCV Ab positive	17	2	15	
HBs Ag, HCV antibody negative	0	0	0	
Healthy volunteers	26	0	26	0%

[a] $p < 0.05$ vs. other groups (χ^2 test).

was then heated at 95° C for 10 min for inactivation of reverse transcriptase, cooled rapidly and stored at −20° C until use for the first PCR.

Sequence of Primers Used in Nested PCR. Sequences of primers used in the experiment were as follows. The primers for albumin gene (11) were 5'-AGAAAGTACCCCAAGTGT-CAA-3' (no. 9) (nucleotides 14997 to 15017), 5'-AGCTGCGA-AATCATCCATAAC-3' (no. 10) (nucleotides 17019 to 16999) for outer primers and 5'-ACTATCTATCCGTGGTCCTGA-3' (no. N1) (nucleotides 15111 to 15115, 15534 to 15549), 5'-TCTTGATTTGTCTCTCCTTCT-3' (no. N2) (nucleotides 15750 to 15730) for inner primers.

The primers for AFP gene (12) were 5'-CTCTTCCAG-CAAAGCACACTTC-3' (no. 7) (nucleotides 15300 to 15320) and 5'-CTCTTCAGCAAAGCAGACTTC-3' (no. 8) (nucleotides 18481 to 18461) for outer primers and 5'-GCTGACAT-TATTATCGGACAC-3' (M1) (nucleotides 16985 to 17005) and 5'-AGCCTCAAGTTGTTCCTCTGT-3' (M2) (nucleotides 18406 to 18386) for inner primers.

We checked integrity of blood RNA by amplifying β-globin mRNA (18) with primers 5'-ACCCAGAGGTTCTTTGAG-TC-3' (no. 26) (nucleotides 295 to 314) and 5'-TCTGATAG-GCAGCCTGCACT-3' (no. 27) (nucleotides 1426 to 1407).

The sense and antisense primers described above were selected from different exons so that we might distinguish amplification of RNA from contaminating DNA.

Nested PCR. Ten microliters of cDNA solution was mixed with 40 μl of the PCR reaction mixture containing 50 mmol/L Tris-HCl (pH 8.3), 44 mmol/L KCl, 1.1 mmol/L $MgCl_2$, 0.013% gelatin, 12.5 pmol of each primer (nos. 7 and 8, 9 and 10 and 26 and 27) and 1 unit *Taq* DNA polymerase (Ampli Taq; Takara Biochemicals). The reaction mixture was overlaid with 50 μl of mineral oil (Sigma Chemical Co., St. Louis, MO) and heated at 93° C for 5 min. It was subjected to a total of 25 cycles of heating at 93° C for 30 sec, 54° C for 45 sec and 72° C for 45 sec with a thermal cycler (GeneAmp PCR system 9600; Perkin-Elmer Cetus, Norwalk, CT). The reaction cycle was terminated by heating at 72° C for 7 min and cooling at 4° C. Five microliters of amplified product using albumin and AFP primer was then used in the second PCR.

Five microliters of the amplified sample was mixed with 5 μl of second PCR buffer (100 mmol/L Tris-HCl (pH 8.3), 500 mmol/L KCl, 10 mmol/L $MgCl_2$, 100 μg/ml gelatin), containing 12.5 pmol of each inner primer (M1 and M2, N1 and N2) and 1 unit of *Taq* DNA polymerase. It was then diluted to a volume of 50 μl with distilled water. The reaction cycle was the same as that used in the PCR, except that annealing temperature was 52° C when N1 and N2 were used as primers.

The PCR products were subjected to electrophoresis on 3% agarose gels and stained with ethidium bromide. The amplified products of albumin, AFP and β-globin were 222, 282 and 283 bp, respectively.

Statistics. Each value is expressed as the mean ± S.E.M. unless otherwise stated. Statistical analysis was performed with the Mann-Whitney U test or the χ^2 test. A p value less than 0.05 was considered significant.

RESULTS

Demonstration of Albumin and AFP mRNA in Liver Tissue and Peripheral Blood. As indicated in Figure 1, albumin mRNA was demonstrated not only in liver tissue and HepG2 cells but also in blood nuclear cells isolated from normal healthy volunteers. In contrast, AFP mRNA was detected in liver tissue and HepG2 cells but not in blood nuclear cells of healthy volunteers.

Detection of Albumin and AFP mRNA in Nuclear Cells of Peripheral Blood from Patients with Liver Diseases. Albumin mRNA was demonstrated in the blood of all normal, healthy volunteers and all patients with liver diseases. In contrast, as indicated in Figure 2 and Table 2, AFP mRNA in peripheral blood was detected in 17 of 33 cases of HCC (52%), 2 of 13 cases of cirrhosis (15%) and 2 of 17 cases of chronic hepatitis (12%) but not in any of 26 healthy volunteers. The detection rate for AFP mRNA in blood was significantly increased in patients with HCC compared with that in patients with cirrhosis or chronic hepatitis, or with normal healthy volunteers (each, $p < 0.05$). However, the detection rate for AFP mRNA in blood was not significantly different among the HCC patients with different serological viral markers.

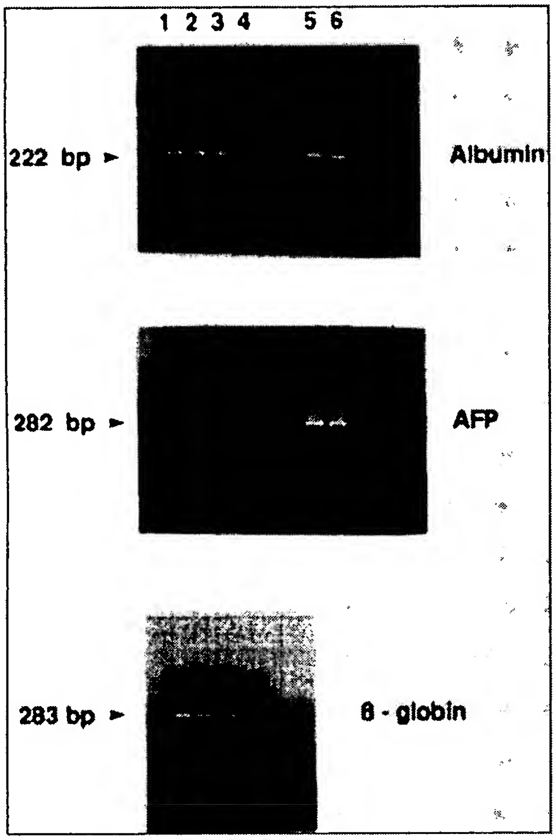


FIG. 1. Albumin and AFP mRNA in nuclear cells of peripheral blood, liver tissue and HepG2 cells as detected with nested RT-PCR. Albumin mRNA was detected in blood, as well as in liver tissue and HepG2 cells, whereas AFP mRNA was detected in liver tissue and HepG2 cells but not in blood. Demonstration of β-globin was performed as a control of RT-PCR. *Lanes 1 to 4:* nuclear cells of peripheral blood *(PBNC)* from healthy volunteers; *lane 5:* liver tissue; *lane 6:* HepG2 cells.

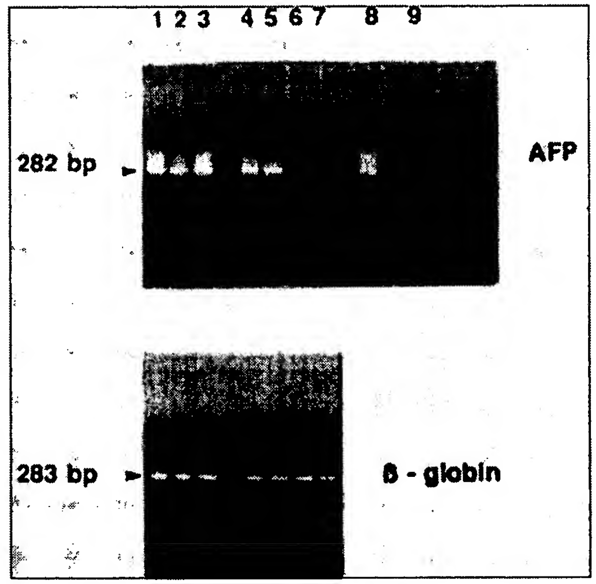


FIG. 2. Demonstration of AFP mRNA in blood of patients with HCC. *Lanes 1 to 3:* HCC with extrahepatic metastasis; *lanes 4 to 7:* HCC without extrahepatic metastasis; *lane 8:* liver tissue (positive control); *lane 9:* negative control of nested RT-PCR (DEPC-treated water was added to sample instead of RNA).

Detection of Albumin and AFP mRNA in the Nuclear Cells of the Blood Collected at Hepatic Vein, Aorta and Inferior Vena Cava. During cardiac catheterization in 15 patients with ischemic heart disease, blood was collected at the different sites. Albumin mRNA was demonstrated in nuclear cells of all the blood collected at different sites. However, as indicated in Table 3, AFP mRNA was detected in five cases at the hepatic vein and in one case at the inferior vena cava. Among these 15 patients, 1 was a patient with cirrhosis, 1 had chronic hepatitis in association with HCV infection and 2 had fatty liver. Among these 4 cases of liver disease, three had AFP mRNA in blood at the hepatic vein, and one patient with cirrhosis only had AFP mRNA in blood from the inferior vena cava. On the other hand, AFP mRNA in blood at hepatic vein was detected in only 2 cases of 11 cases without abnormal liver function, but AFP mRNA was not demonstrated in the blood at aorta and inferior vena cava in these 11 cases.

Characteristics of the HCC Patients with AFP mRNA in Peripheral Blood. Characteristics of the hepatocellular carcinoma patients with AFP mRNA in peripheral blood were clarified in relation to tumor size, number of tumor foci, total volume of tumor tissue in the liver, serum AFP concentration and presence of extrahepatic metastases.

In the cases with several tumor foci in the liver, the largest tumor was noted. AFP mRNA in blood was detected in seven of eight cases (87%) of HCC showing diffuse or large tumors occupying both lobes of the liver. As indicated in Figure 3, tumor diameter in the liver was significantly increased in the HCC patients with AFP mRNA in blood (8.1 ± 4.2 cm in diameter), when compared with the patients without AFP mRNA in the circulation (3.6 ± 2.8 cm in diameter) ($p < 0.05$). When the patients were divided into two groups according to diameter of tumor foci, the incidence of AFP mRNA in blood was markedly increased in the patients with HCCs 5 cm or more in diameter (14 of 17 cases [82%]) compared with those less than 5 cm in diameter (3 of 16 cases [19%]) ($p < 0.05$; χ^2 test).

Although the number of tumor foci was not significantly different between the groups with AFP mRNA detected and undetected group (3.8 ± 2.3 vs. 3.3 ± 2.1 foci, respectively) (Fig. 4), AFP mRNA in blood was detected in 9 of 15 cases (60%) of HCCs showing four or more tumor foci in the liver. In contrast, AFP mRNA was detected in 8 of 18 cases (44%) with fewer than 4 tumor foci (Fig. 4). Although there is a tendency toward an increased detection rate of AFP mRNA in the peripheral blood of patients with four or more tumor foci, it is not significantly compared with cases with fewer than four tumor foci in the liver.

We calculated total volume of tumor tissue by adding

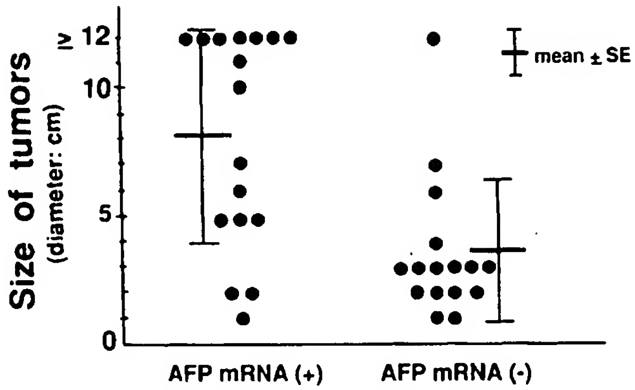


FIG. 3. Characteristics of patients with HCC in relation to size of tumor foci in the liver. Tumor size in the patients with AFP mRNA in blood was significantly larger than in those without AFP mRNA in blood ($p = 0.041$; Mann-Whitney U test).

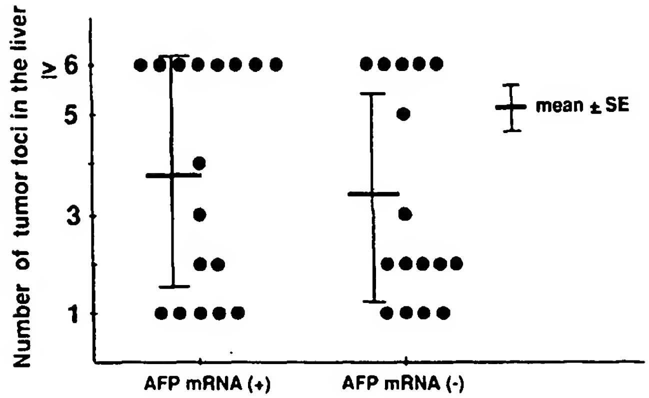


FIG. 4. Characteristic of patients with HCC in relation to the number of tumor foci in liver. There was a tendency toward an increased number of tumor foci in the patients with AFP mRNA in blood compared with that in patients without AFP mRNA, but it was not significant ($p = 0.584$, Mann-Whitney U test).

TABLE 3. Detection of AFP mRNA in blood collected at different sites

Patient no.	Hepatic vein	Aorta	Inferior vena cava	Liver disease
1	+	ND	+	Alcoholic cirrhosis
2	+	–	–	Chronic hepatitis (HCV antibody positive)
3	+	–	–	Fatty liver (ALT, 50 U/L)
4	–	–	–	Fatty liver (ALT, 45 U/L)
5	+	–	–	–
6	+	–	–	–
7	–	–	–	–
8	–	–	–	–
9	–	–	–	–
10	–	–	–	–
11	–	–	–	–
12	–	–	–	–
13	–	–	–	–
14	–	–	–	–
15	–	–	–	–

ND, not determined.

all tumor volume detected with ultrasonography, computed tomography or both. As indicated in Figure 5, total volume of tumor tissue in the patients with AFP mRNA in blood (441 ± 103 cm^3) was significantly larger than those in patients without AFP mRNA in the circulation (87 ± 49 cm^3) ($p < 0.05$).

AFP concentration in serum was significantly increased in HCC patients with AFP mRNA in blood (62,738 ± 7,031 ng/ml) compared with patients without AFP mRNA in peripheral blood (536 ± 240 ng/ml) (Fig. 6) ($p < 0.05$; Mann-Whitney U test).

Extrahepatic metastasis was detected by means of imaging techniques such as scintigraphy or computed tomography or on postmortem examination. Extrahepatic metastases were demonstrated in 6 patients among 17 cases with AFP mRNA but not in 16 patients without AFP mRNA in peripheral blood ($p < 0.05$; χ^2 test) (Fig. 6). When the patients were classified according to the presence or absence of extrahepatic metastases, AFP mRNA was detected in the blood of all six patients with extrahepatic metastasis, in contrast to 11 of 27 patients without extrahepatic metastases ($p < 0.05$; χ^2 test) (Table 4).

DISCUSSION

Because metastasis of carcinoma is one of the most important factors affecting prognosis, extensive trials to detect cancer metastasis have been carried out with tumor-associated genes or proteins. Hematogenous metastases of colon cancer and melanoma are demonstrated by staining of the tumor-specific epithelial cytokeratin protein (cytokeratin 18) in bone marrow (19) and the presence of transcription of tyrosinase gene in the circulation (20), respectively. In addition, tumor-specific genes such as bcr-abl hybrid for chronic myelogenous leukemia (7) and mutation of K-*ras* codon 12 for pancreatic cancer (8) are also used for detection of tumor cells in the circulation.

The presence of circulating tumor cells is known to be an indicator of hematogenous spread of tumor cells

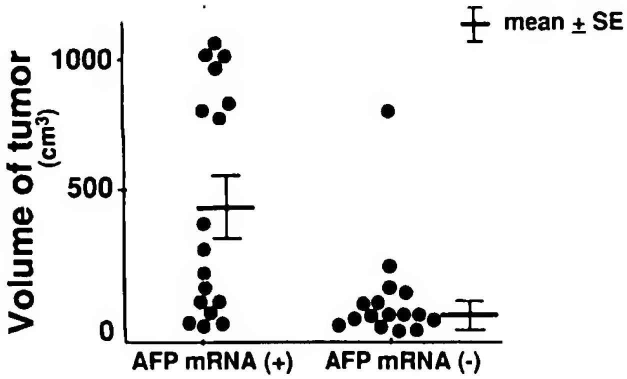


FIG. 5. Characteristics of patients with HCC in relation to total volume of tumor tissue. Total volume of tumor tissue in patients with AFP mRNA in blood was significantly larger than that in patients without AFP mRNA in blood ($p = 0.009$, Mann-Whitney U test).

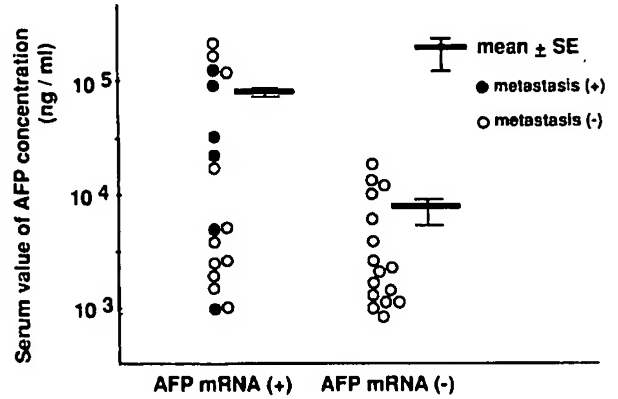


FIG. 6. Characteristics of patients with HCC in relation to serum AFP concentration. Serum AFP concentration was significantly increased in the patients with AFP mRNA in blood compared with patients without AFP mRNA in blood ($p = 0.040$, Mann-Whitney U test). Extrahepatic metastasis was detected in 6 of 17 patients with AFP mRNA but not in the patients without showing AFP mRNA in peripheral blood ($p < 0.05$, χ^2 test). Each S.E.M. value was calculated with the arithmetic mean instead of the geometric mean. Solid circles denote patients with extrahepatic metastasis; open circles denote patients without.

leading to metastasis at other organs. An enhanced serum AFP concentration is used for a screening of HCC (16) but does not reflect the presence of circulating tumor cells. Therefore an increased AFP concentration in serum cannot directly predict metastasis of HCC. In this study, we tried to demonstrate circulating HCC cells, which may be released from tumor foci into the circulation, by detecting the hepatocyte- or HCC-associated mRNA in the nuclear cell component of peripheral blood using nested RT-PCR because the number of circulating malignant cells is too low. In the preliminary experiments, we tried to demonstrate circulating HCC morphologically after collecting nuclear cells with a cytospinner and staining them by use of immunohistochemistry but failed.

Because albumin is known to be produced by hepatocytes (9), we tried to examine the presence of albumin mRNA in peripheral blood of normal healthy volunteers as a negative control. However, albumin mRNA was detected not only in liver tissue and HepG2 cells but also in the nuclear cell component of peripheral blood from normal volunteers on nested RT-PCR, suggesting that tiny amounts of albumin message are transcribed in nuclear blood cells such as lymphocytes and neutrophils. These results are consistent with the report of McLeod et al. (21), which indicated that albumin mRNA is detected not only in the liver but in several other organs—such as testis, uterus, placenta and yolk sac in rats—by RT-PCR. Therefore it is not rational to use albumin mRNA for detecting a small amount of circulating HCC with nested RT-PCR, and these results could prove wrong an earlier report stating that transcription of albumin mRNA is a marker of the presence of HCC cells (22). Although the albumin mRNA may be transcribed by neutrophils, lymphocytes or both, there is an another possibility: that there are circulating hepatocytes expressing albumin mRNA, but that the lack of AFP mRNA in peripheral blood of such individual may be due to the inability of normal hepatocytes to transcribe AFP mRNA.

We, then, sought AFP mRNA for detection of HCC in peripheral blood. AFP mRNA was detected in liver tissue and HepG2 cells but not in peripheral blood, even on nested RT-PCR. Because AFP mRNA was not demonstrated in the blood of normal healthy volunteers, AFP mRNA in peripheral blood may have indicated the presence of hepatocytes/HCC cells in the circulation. AFP mRNA was detected in the circulation of 52% of patients with HCC and in that of 15% and 12% of patients with cirrhosis and chronic hepatitis, respectively. The presence of AFP mRNA in a small number of patients with chronic hepatitis or cirrhosis may have been related to the fact that increased serum AFP concentrations are found in patients recovering from acute hepatitis or acute exacerbation of chronic hepatitis (23) and that a few injured or necrotic hepatocytes may have entered the circulation in these cases. To approach this possibility, we collected blood at different sites during cardiac catheterization. AFP mRNA in the blood of hepatic vein was demonstrated in 3 of 4 patients with liver diseases but in only 2 of 11 cases without liver diseases. However, AFP mRNA in the blood of inferior vena cava was detected in one case with cirrhosis only. These data suggest that injured or necrotic hepatocytes released into the circulation may be detected in blood taken at the hepatic vein. Because these cells might be removed from the circulation by macrophages in the lung, AFP mRNA was not any more detected at aorta or at inferior vena cava. However, many injured hepatocytes were released from the liver and spilled into the circulation after passage through the lung, AFP mRNA could be detected in peripheral blood.

We then examined the characteristics of the patients with HCC who showed AFP mRNA in peripheral blood.

TABLE 4. Characteristic of patients with HCC in relation to extrahepatic metastasis

Metastasis	AFP mRNA in peripheral blood			
	No.	Positive	Negative	% Positive
Present	6	6	0	100[a]
Absent	27	11	16	41

[a] $p < 0.05$ vs. those without metastasis (χ^2 test).

The incidence of AFP mRNA in blood was significantly increased in association with the size of tumors and serum AFP concentration. In addition, extrahepatic metastasis was demonstrated only among the patients showing AFP mRNA, not in the patients without AFP mRNA in peripheral blood. These results may have indicated that tumor cells were released from tumor foci into the circulation when tumor size increased. Furthermore, detection of AFP mRNA in blood may reflect the presence of HCC/hepatocytes in the circulation, which could lead to extrahepatic metastasis. On the other hand, serum AFP levels may not reflect the existence of extrahepatic metastasis but may be useful only in screening of HCC (16).

AFP mRNA was found in the peripheral blood of all cases (100%) of HCC with extrahepatic metastases and in 41% of the patients without extrahepatic metastases. These results could indicate that detection of AFP mRNA in peripheral blood is a useful marker to select the patients in whom extrahepatic metastases might develop, although further study of these patients will be necessary to determine whether extrahepatic metastases develop in the near future.

The efficacy of tumor metastasis is considered to depend on the balance between selected properties of tumor cells and reactivity of the host (24). In the process of metastasis, tumor cells are scattered from the original site, spreaded hematogenously, arrested at the small vessels and extravasated from the vessels. During these processes, tumor cells are attacked and destroyed by immunologically responsive cells (25) or by mechanical force. Thus, a moderate number of circulating tumor cells is needed to form foci of micrometastasis *in vivo* (26). Therefore the detection of cancer cells in blood does not always reflect the existence of metastatic foci. Moreover, metastatic foci at other organs may be too small to be detected, in some cases, with the imaging techniques used. Because the presence of micrometastases of HCC at other organs leads to poor prognosis in patients with HCC even after transplantation of the liver (6), prediction of micrometastasis on the basis of detection of circulating HCC (AFP mRNA in blood) before liver transplantation could be a useful tool for the selection of transplant recipients.

Acknowledgment: We thank the members of the cardiovascular section of our department for their aid in collecting blood during cardiac catheterization.

REFERENCES

1. Oka H, Kurioka N, Kim K, Kanno T, Kuroki T, Mizokuchi Y, Kobayashi K. Prospective study of early detection of hepatocellular carcinoma in patients with cirrhosis. HEPATOLOGY 1990;12: 680-687.
2. Tsuzuki T, Ogata Y, Iida S, Shimazu M. Hepatic resection in 125 patients. Arch Surg 1984;119:1025-1032.
3. O'Halpin D, Legge D, MacErlean DP. Therapeutic arterial embolization: report of five years' experience. Clin Radiol 1984; 35:85-93.
4. Shiina S, Tagawa K, Niwa Y, Unuma T, Komatsu Y, Yoshiura K, Hamada E, et al. Percutaneous ethanol injection therapy for hepatocellular carcinoma. Am J Roentogenol 1993;160:1023-1028.
5. Lee Y, Teer DA. Primary liver cancer: pattern of metastasis. J Surg Oncol 1987;36:26-31.
6. Ringe B, Wittekind C. The role of liver transplantation in hepatobiliary malignancy: a retrospective analysis of 95 patients with particular regard to tumor stage and recurrence. Ann Surg 1989;209:88-98.
7. Hirai H, Ishikawa F. Analysis of Ph1-positive leukemia by PCR [in Japanese]. Rinsho-Ketsueki 1991;32:319-326.
8. Tada M, Omata M, Kawai S, Saisho H, Ohto M, Sakai RK, Sninsky JJ. Detection of ras gene mutation in pancreatic juice and peripheral blood of patients with pancreatic adenocarcinoma. Cancer Res 1993;53:2472-2474.
9. Weiner FR, Czaja MJ, Giambrone MA, Wu CH, Wu GY, Zern MA. Development of molecular hybridization technology to evaluate albumin and procollagen mRNA content in baboons and man. HEPATOLOGY 1987;7:19S-25S.
10. Tamaoki T, Fausto N. Expression of the α-feto protein gene during development, regeneration and carcinogenesis. In: Stein G, Stein J, eds. Recombinant DNA and cell proliferation. New York: Academic Press, 1984:145-168.
11. Minghetti PP, Ruffner DE, Kuang WJ, Dennison OE, Hawkins JW, Beattie WG, Dugaiczyk A. Molecular structure of the human albumin gene is revealed by nucleotide sequence within q11-22 of chromosome 4. J Biol Chem 1986;261:6747-6757.
12. Sakai M, Morinaga T, Urano Y. The human α-fetoprotein gene: sequence organization and the 5'flanking region. J Biol Chem 1985;260:5055-5060.
13. Tsutsumi T, Nakao K, Mitsuoka S, Hamasaki K, Tsuruta S, Shima M, Nakata K. Regulation of albumin and α-feto protein gene expression by colloid osmotic pressure in human hepatoma cells. Gastroenterology 1993;104:256-262.
14. Camper SA, Godbout R, Tilghman SM. The developmental regulation of albumin and α-fetoprotein gene expression. Prog Nucleic Acid Res Mol Biol 1989;36:131-143.
15. Peng SY, Lai OL, Chu JS, Lee PH, Tsung PT, Chen DS, Hsu HC. Expression and hypomethylation of α-fetoprotein gene in unicentric and multicentric human hepatocellular carcinoma. HEPATOLOGY 1993;17:35-41.
16. Okuda K, Kotoda K, Obata H, Hayashi N, Hisamitsu T. Clinical observations during a relatively early stage of hepatocellular carcinoma, with special reference to serum α-fetoprotein levels. Gastroenterology 1975;69:226-234.
17. Pinthanond D, Petchclai B. A preserved leukocyte preparation for quality control. Am J Clin Pathol 1975;63:32-34.
18. Lawn RM, Efstratiadis A, O'Connel C, Maniatis T. The nucleotide sequence of the human β-globin gene. Cell 1980;21:647-651.

19. Lindemann F, Schlimok G, Dirschedl P, Witte J, Riethmuller G. Prognostic significance of micrometastatic tumor cells in bone marrow of colorectal cancer patients. Lancet 1992;340:685-688.
20. Smith B, Selby P, Southgate J, Pittman K, Bradley C, Blair GE. Detection of melanoma cells in peripheral blood by means of reverse transcriptase and polymerase chain reaction. Lancet 1991;338:1227-1229.
21. McLeod JF, Cooke NE. The vitamin D–binding protein, α-feto protein, albumin multigene family: detection of transcripts in multiple tissues. J Biol Chem 1989;264:21760-21769.
22. Carr BI, Kar S. An assay for hepatoma micrometastasis: albumin gene expression in peripheral blood from patients with advanced hepatocellular carcinoma (HCC) [Abstract]. HEPATOLOGY 1991;14: 108A.
23. Bloomer JR, Waldmann TA, McIntire KR, Klatskin G. α-feto-protein in nonneoplastic hepatic disorders. JAMA 1975;233: 38-41.
24. Paul BU, Lee CL. Organ preference of metastasis. The role of organ-specifically modulated endothelial cells. Lab Invest 1988; 58:379-387.
25. Shiratori Y, Nakata R, Okano K, Komatsu Y, Shiina S, Kawase T, Sugimoto T, et al. Inhibition of hepatic metastasis of colon carcinoma by asialo GM_1–positive cells in the liver. HEPATOLOGY 1992;16:469-478.
26. Tominaga T, Yoshida Y, Kitamura M, Kosaki G. Liver metastasis of colon 26 cells implanted into the superior mesenteric vein in mice. Jpn J Cancer Res 1987;78:846-850.

Vol. 207, No. 2, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 15, 1995 Pages 813-818

J

SENSITIVE ASSAY FOR DETECTION OF HEPATOCELLULAR CARCINOMA ASSOCIATED GENE TRANSCRIPTION (ALPHA-FETOPROTEIN mRNA) IN BLOOD

Masayuki Matsumura [1)*], Yasuro Niwa [1)], Yohko Hikiba [2)], Ken-ichi Okano [2)], Naoya Kato [1)], Shuichiro Shiina [1)], Yasushi Shiratori [1)] and Masao Omata [1)]

1) Second Department of Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan 113,

2) Div. of Gastroenterology and Hepatology, Institute of Adult Disease, Asahi Life Foundation, Nishi-Shin'juku, Shin'juku, Tokyo, Japan

Received January 6, 1995

SUMMARY: The sensitivity of RT-PCR and nested RT-PCR for detection of circulating hepatocellular carcinoma was assessed by demonstrating the tumor cell-associated gene transcription, alpha-fetoprotein mRNA, in the nuclear cells of peripheral blood. When HepG2 cells were mixed in blood, 100-1000 tumor cells/5ml of blood could be detected by RT-PCR, in contrast to 1-10 tumor cells/5ml of blood by nested RT-PCR. In addition, $2x10^4$ copies of AFP mRNA were found in one HepG2 cell when analyzed by the quantitative nested RT-PCR assay. Thus, the nested RT-PCR assay could provide a useful tool for detecting a tiny amount of circulating tumor cells in patients with hepatocellular carcinoma presenting extra-hepatic metastasis.

Prognosis of patients with hepatocellular carcinoma (HCC) is estimated by several factors, such as histological differentiation of tumor cells, extent of tumor size, and extent of lymphatic or hematogenous spread. Venous invasion of tumor cells, first step to induce blood-born metastasis, is one of the important factor affecting prognosis of these patients. Although the efficacy of tumor metastasis is considered to depend on the balance between selected properties of tumor cells and reactivity of the host against tumor cells. In the process of metastasis, tumor cells are scattered from the original site, spreaded hematogenously and arrested at small vessels. Thus, detection of tumor cells in the circulation might predict metastasis of tumor. However, the number of

*Correspondence. Fax:03-3814-0021.

0006-291X/95 $5.00

tumor cells in circulation could be extremely too small to be detected morphologically.

Recently, tumor associated gene in the circulating tumor cells (reflecting the presence of tumor cells in the circulation) could be detected by PCR in patients with leukemia (1), or in patients with pancreatic cancer with distant metastasis (2). Thus, detection of hepatocellular carcinoma associated gene in the circulation might be related to the hematogenous spreading metastasis of HCC, even though overt metastasis could be obscure.

In the present study, the difference of sensitivity among RT-PCR and nested RT-PCR assay for detection of tumor cells in blood has been estimated.

MATERIALS AND METHODS

Cell line; HepG2 cell line (3) (a gift from Dr. Makoto Noda in Riken Cell Bank; RCB 459) was maintained in Dulbecco's Minimum Essential Medium (Gibco-BRL, Grand Island, NY) containing 10% fetal bovine serum (Gibco-BRL).

Peripheral blood from healthy volunteer was collected in a disposable syringe with 0.1% EDTA. One thousand HepG2 cells were added into 5ml of blood, and serial dilution of tumor cells was made. Each dilution sample contained 1,000, 100, 10 and 1 HepG2 cells per 5ml of blood.

Preparation of nuclear cells from peripheral blood; After adding one ml of 5% dextran-saline solution into 5 ml of blood (4), the syringe was stood still for 30 min at room temperature. Supernatant was collected and centrifugated at 500 x g for 25 min. Residual erythrocytes was lysed by adding distilled water and isotonicity was then restored after 25 sec by adding the same volume of 1.8% NaCl solution. After centrifugation at 350 x g for 5 min, the cells were immediately frozen using liquid nitrogen and stored at -80°C until use.

Extraction of RNA and synthesis of complementary DNA (cDNA); By using RNAsol B (BIOTECK LABOLATORIES, Houston, TX), total RNA was extracted from nuclear cell component of peripheral blood. About 5 μg of RNA was extracted from 5 ml of blood. In addition, one μg of RNA was extracted from 5×10^4 HepG2 cells.

One μg of RNA, which was heated at 95°C for 5 min and rapidly cooled on ice, was added in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.25 μg random primer (hexamer) (Takara Biochemicals, Kyoto, Japan), 18 units RNAse inhibitor (Takara Biochemicals), and 200 units M-MLV reverse transcriptase (Gibco-BRL). The volume was adjusted to 10 μl by adding DEPC treated water. Complementary DNA (cDNA) was synthesized by incubating the mixture at 42 °C for 60 min, and it was subsequently heated at 95°C for 5 min to inactivate reverse transcriptase. Complementary DNA was kept at -20°C until use.

Sensitivity of RT-PCR and nested RT-PCR

Polymerase chain reaction (PCR); Primers of AFP (5) mRNA used in RT-PCR were 5'- CTCTTCCAGCAAAGCACACTTC -3' (#7: upper stream) and 5'-CTCTTCAGCAAAGCAGACTTC -3' (#8: down stream). Integrity of RNA

was checked by amplifying ß-globin (6) mRNA with primers 5'-ACCCAGAGGTTCTTTGAGTC -3' (#26: upper stream) and 5'-TCTGATAGGCAGCCTGCACT - 3' (# 27: down stream).

Ten μl of cDNA solution was mixed with 40 μl of PCR reaction mixture containing 50 mM Tris-HCl (pH 8.3), 44 mM KCl, 1.1 mM $MgCl_2$, 0.013% gelatin, 12.5 pmol each primer (#7 and #8, or #26 and #27) and 1 unit Taq DNA polymerase (Takara Biochemicals). After initial denaturation at 95°C for 5 min, PCR was performed according to the temperature profile (93°C for 30 sec, 54°C for 45 sec and 72°C for 45 sec) for 35 cycles. The reaction was terminated by heating at 72°C for 7 min and cooled to 4°C.

Nested RT-PCR; Primers of AFP mRNA used in the nested PCR were 5'-GCTGACATTATTATCGGACAC-3' (#M1: upper stream) and 5'-AGCCTCAAGTTGTTCCTCTGT-3' (# M2: down stream).

Five μl of the amplified product of the RT-PCR was mixed with 5 ul of second PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 10 mM $MgCl_2$, 100 μg/ml gelatin), 12.5 pmol of each primer (# M1 and # M2) ,1 unit of Taq DNA polymerase and diluted to 50μl with distilled water. Themperature profile of nested PCR was the same one as described above.

Gel electrophoresis; Amplified product was electrophoresed on 3% agarose gels and stained with ethidium bromide. Size of the amplified product of AFP mRNA was 359 bp (PCR), 282 bp (nested PCR), and 283 bp for ß-globin.

Quantification of AFP mRNA in HepG2 cell

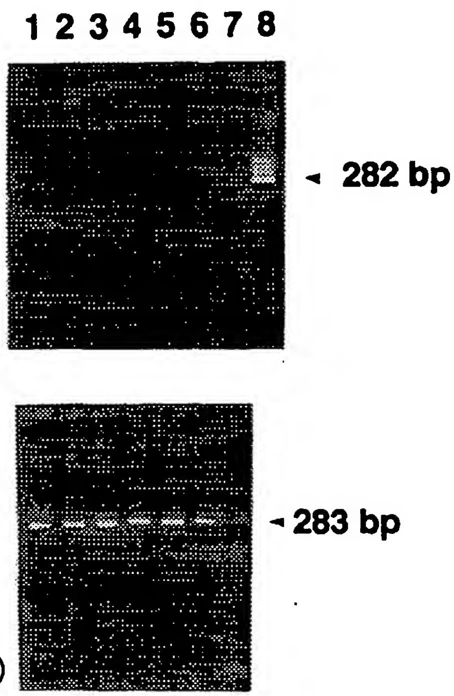
Synthesis of internal standard; RNA was extracted from HepG2 cells and was applied to RT-PCR. New restriction site (cut by Hind III) was introduced in the middle of amplified product according to the method described by Horton and Pease (7). The product was then inserted into pBluescript-II vector under the promoter of T7 RNA polymerase, and its sequence was confirmed by the ALF DNA Sequencer (Pharmacia, Uppsala, Sweden). After linearizing the plasmid, RNA was synthesized using T7 RNA polymerase. The RNA was quantified and diluted serially with DEPC-treated water. It was used as internal standard of quantitative PCR assay.

After adding the serially-diluted (10^4 to 10^{10} copies) internal standards to each of 1 μg RNA extracted from HepG2 cells, the mixture was reverse transcribed followed by nested PCR as described above. Amplified product was then cut with Hind III and was electrophoresed on 3% agarose gel. Three bands of 282 bp (wild type), 170 and 112 bp (both are derived from the internal standard) were demonstrated. An amount of AFR mRNA was calculated by comparison the intensity of the band of wild type with those of internal control (summation of two bands) (Figure 3).

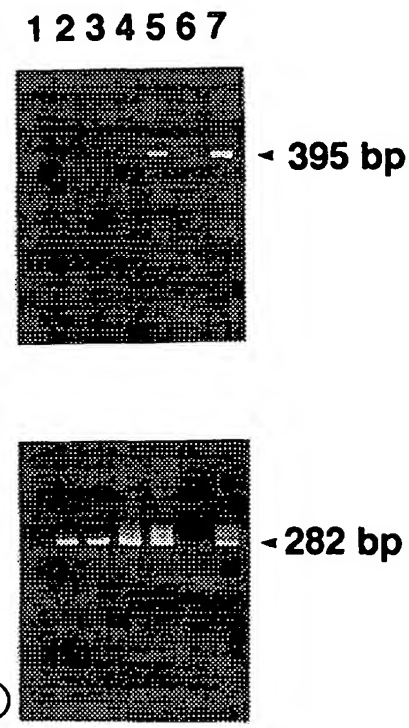
RESULTS

Absence of AFP mRNA in blood of healthy volunteers; AFP mRNA was not demonstrated in nuclear cell component of peripheral blood of healthy volunteers both by RT-PCR and even by nested RT-PCR (Fig. 1).

Detection of AFP mRNA in diluted samples of HepG2 cells; As shown in Fig.



1



2

Fig.1. Amplification of AFP mRNA in blood of healthy volunteers.
Upper panel shows the result of nested PCR with primers #M1 and #M2 (AFP). Amplified product of PCR is 282 bp.
Lower panel shows the result of PCR with primers #26 and #27 (ß-globin). Amplified product is 283 bp.
Lanes 1-6: blood from healthy volunteers, lane 7: negative control (without RNA), lane 8: positive control (RNA from HepG2 cells).

Fig.2. Sensitivity of RT-PCR and nested RT-PCR for detection of HepG2 cells in blood.
Lanes 1-5: Dilution of HepG2 cells. Each lane includes (from left to right) 0, 1, 10, 100 and 1,000 HepG2 cells in 5 ml of normal blood, respectively, lane 6: negative control (without RNA), lane 7: positive control (1 μg of RNA from HepG2 cells).

2, AFP mRNA was detected in 5 ml of blood containing 100 tumor cells by the RT-PCR. In contrast, AFP mRNA was demonstrated in 5 ml of blood containing 1-10 tumor cells only by the nested RT-PCR. The sensitivity of nested RT-PCR was enhanced by around 10-100 folds, as compared with RT-PCR only.

Absolute number of AFP mRNA; As indicated in Fig. 3, 10^9 copies of AFP mRNA were found in 1 μg RNA of HepG2 cells. As one μg RNA was extracted from $5x10^4$ HepG2 cells, around $4x10^3$ copies of AFP mRNA were found in one HepG2 cell.

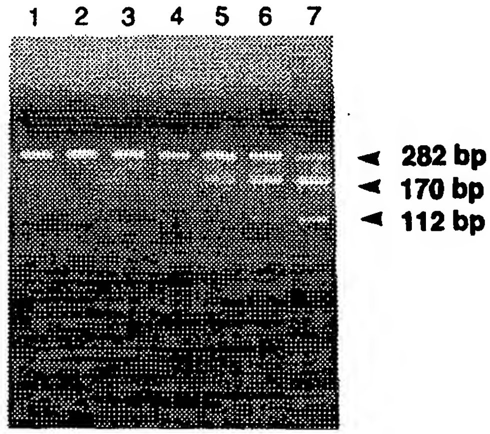


Fig.3. Quantification of AFP mRNA in HepG2 cell.
Lanes 1-7: Each lane includes the serially diluted-internal standard; (from left to right) 10^4, 10^5, 10^6, 10^7, 10^8, 10^9 and 10^{10} copies per sample.

DISCUSSION

In order to detect tumor cells in blood, demonstration of tumor associated gene has been developed using molecular techniques (1,2). In the recent study, albumin mRNA in blood has been found as a marker of circulating hepatocytes (8). There are some papers indicating that the production of AFP is increased in hepatocellular carcinoma cell lines such as HepG2 (3), and expression of AFP gene is reported to be increased during carcinogenesis (9). Thus, we have developed PCR assay for detection of HCC-associated tumor gene transcript, AFP mRNA, in nuclear cell component of blood, since the number of circulating tumor cells in patients with HCC demonstrating metastasis at distant organs might be extremely too small. In order to elucidate the sensitivity of RT-PCR and nested RT-PCR for detection of HCC cells in blood, HepG2 cells were mixed with blood, as HepG2 cells were established from hepatoblastoma and produces adequate amount of AFP (3). AFP mRNA could be detected by RT-PCR when 100 tumor cells are present in 5 ml of blood, in contrast to 1-10 tumor cell per 5 ml of blood by nested RT-PCR, suggesting that the sensitivity of nested RT-PCR is around 100 folds enhanced as compared with RT-PCR. Thus, if HepG2 cells were circulating in a body (total volume of blood; around 5,000 ml), their presence could be detected by RT-PCR when more than 10^5 tumor cells present in circulation. On the other hand, AFP mRNA could be detected by nested RT-PCR even when only 10^3 tumor cells are present in the circulation. Although a moderate number of circulating tumor cells might be present as assessed from the calculation of detection of HepG2 cells, there might be several steps for establishment of metastasis (10) such as adhesion of tumor cells at

vascular endothelium, migration into the extracellular space, and proliferation at the metastatic foci. During these process, immunological reaction of host organs takes place, and some tumor cells might be damaged by immunocompetent cells or by mechanical force of blood flow or by platelet aggregation.

Furthermore, the quantitative analysis has been performed by competitive RT-PCR (11) to estimate an amount of AFP mRNA in HepG2 cell, indicating that around $2x10^4$ copies of AFP mRNA were included in one HepG2 cell. As one HepG2 cells per 5 ml of blood could be detected by this nested RT-PCR, around 4,000 copies of AFP mRNA in one ml of blood could be demonstrated by this assay.

These results suggest that high sensitivity detection assay of nested RT-PCR could be useful for demonstration of hematogenous spreading tumor cells. In addition, further prospective study is needed for prediction of metastasis in patients with HCC by detecting circulating tumor cells.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Ministry of Health and Welfare; the Ministry of Education, Culture and Science, Japan.

REFERENCES

1. Hirai, H., Ishikawa, F. (1991) Rinsho-Ketsueki (in Japanese) 32, 319-326.
2. Tada, M., Omata, M. (1993) Cancer Res 53, 2472-2474.
3. Aden, D.P., Fogel, A. (1979) Nature 282, 615-616.
4. Pintthanond, D. and Petchclai, B. (1975) American Journal of Clinical Pathology 63, 32- 34.
5. Sakai, M., Morinaga, T. (1985) J Biol Chem 260, 5055-5060.
6. Lawn, R. M., Efstratiadis, A. (1980) Cell 21 , 647-651.
7. Horton, R.M., Pease, L.R. (1991) In Directed Mutagenesis. (McPherson, M.J. Ed.) pp.217- 247. Oxford University Press, Oxford.
8. Hillaire, S., Barbu, V. (1994) Gastroenterology 106, 239-242.
9. Tamaoki, T, Fausto, N. (1984) Recombinant DNA and cell proliferatiion. (Stein, G. Stein, J. Ed.) pp 145-168. Academic Press, New York.
10. Hart, I.R., Saini, A. (1992) Lancet 339, 1453-1457.
11. Becker-Andre, M., Hahlbrock, K. (1989) Nucleic Acids Res 22, 9437-9446.

K

Isolating RNA from whole blood — the dawn of RNA-based diagnosis?

Donald E. Macfarlane and Christopher E. Dahle

A novel cationic surfactant solution is used to lyse blood cells and precipitate RNA and DNA. The RNA is recovered by extracting the pellet with a small volume of guanidinium isothyocyanate or formamide.

THE addition of a novel cationic surfactant (Catrimox-14) solution to whole blood results in lysis of the cells and the precipitation of RNA and DNA complexed with the surfactant. After centrifugation, the RNA can be recovered from the pellet by either extraction with hot buffered formamide followed by ethanol precipitation, or with guanidinium isothiocyanate followed by extraction at slightly acid pH and isopropanol precipitation. This simple and rapid method yields RNA that is suitable for analysis by reverse transcriptase and the polymerase chain reaction (RT-PCR) without further purification. Compared with methods that require prior isolation of leukocytes or pathogens from blood, isolating RNA from blood directly increases the feasibility of using RNA-based diagnosis of infections, inflammatory diseases, leukaemia and inherited disorders.

DNA is not an ideal target for molecular diagnosis: most eukaryotic DNA consists of non-informative sequences between genes, and of introns within them. Compared with DNA, analysing RNA can be more informative because the level of expression of many genes reveals the activity of the cell as well as its genetic origin, and because the cell usually contains multiple copies of RNA sequences of interest. The utility of RNA-based diagnosis has been restricted by the difficulty in extracting RNA from clinical specimens before the RNA is degraded.

Recent advances in the analysis of RNA, particularly the use of RT-PCR[1], have greatly increased the information that can be derived from small amounts of RNA even when it is partially degraded. Traditional northern blots are performed with about 10 μg of undegraded RNA, preferably using RNA enriched with mRNA. RT-PCR can detect a few hundred copies of a sequence of RNA, and typical analyses can be performed with much less than 1 μg of cellular RNA.

Widely used methods of extracting RNA from cells depend on the ability of chaotropic concentrations of guanidinium isothiocyanate (often with an anionic surfactant) to lyse the cells and simultaneously inhibit RNase. This results in a complex solution of DNA, RNA, lipid, small molecules, and protein. The RNA can be recovered from this mixture by ultracentrifugation, or the DNA, protein, and most other molecules can removed by phenol extraction at slightly acid pH, followed by precipitating the RNA with an alcohol[2]. Although such methods can be applied to whole blood, researchers studying the RNA of blood leukocytes or blood-borne pathogens have generally preferred to use centrifugal procedures to isolate the cells or pathogens from the blood sample before extracting the RNA. This may be to avoid the inhibitors of PCR found in blood (such as porphyrins and haematin[3]). Unfortunately, it is often not practical to perform cell separating procedures in a timely fashion in a clinical environment. Because some species of RNA (especially those encoded by some oncogenes and cytokines) have a short half-life in the cell, there are advantages in mixing the blood sample with an RNA extractant as soon as it is drawn. The extractant must be capable of inhibiting RNase, as blood contains enough RNase to destroy extracellular RNA in a few seconds[4].

Cationic surfactants

The ability of cationic surfactants to precipitate RNA and DNA was reported a generation ago[5,7]. This precipitation is probably due to the formation of electrically

TABLE 1*a* Pellet size after lysis of blood

	12-TMA	14-TMA	16-TMA	12-BA	14-BA	16-BA	18-BA
Bromide	2	2	2				
Chloride	1	2	1	2	2	(2)	(5)
Phosphate	2	2	1	2	1	(1)	(1)
Sulphate	0	1	1	2	2	2	(2)
Formate	3	0	2	1	0	(0)	1
Acetate	0	0	1	0	0	(0)	1
Propionate	3	0	2	1	0	0	0
Oxalate	1	1	1	3	3	3	(2)
Malonate	0	1	1	3	3	2	(2)
Succinate	1	1	1	3	3	(2)	(2)
Citrate		3	3				

TABLE 1*b* Precipitation of RNA (per cent)

	12-TMA	14-TMA	16-TMA	12-BA	14-BA	16-BA	18-BA
Bromide	91	88	79				
Chloride	95	96	70	77	78	68	57
Phosphate	94	95	87	86	86	94	88
Sulphate	101	102	100	76	82	83	87
Formate	44	28	80	33	40	94	86
Acetate	15	30	32	33	31	90	76
Propionate	68	24	82	24	30	66	70
Oxalate	102	99	101	85	75	72	68
Malonate	102	92	93	95	94	80	87
Succinate	100	91	91	93	90	82	89

METHODS. Blood (200 μl, anticoagulated with citrate), was added to 1 ml of the indicated surfactant (0.1 M), together with 50 μl phosphate buffered saline containing 10 μg tRNA as carrier and 20,000 c.p.m. ^{32}P-RNA (a 2,000-base transcript). One hour later, the mixture was centrifuged, and the supernatant was aspirated and discarded. The pellet was examined visually and graded (Table 1*a*) as to its size and colour as follows: 0 = pellet almost invisible to the unaided eye; 1 = lightly coloured pellet or smear of material on side of tube with minimal volume; 2 = brown pellet 2–3 mm in long axis, incompletely covering the bottom of the tube; 3 = brown pellet 3–4 mm in long axis, completely covering bottom of tube; 4 = dark brown, 4–5 mm in long axis; 5 = greater than 5 mm in long axis. Some surfactant solutions tended to crystallize on storage at room temperature. In these cases, the results are given in parentheses. The pellet was then dissolved with 100 μl formamide containing 0.3 M sodium acetate and 50 mM acetic acid by heating and vortexing. The radioactivity was then estimated by scintillation counting, and is expressed as a percentage of the added radioactivity (Table 1*b*). The abbreviations are 12-, 14-, or 16-TMA:- acyltrimethylammonium with acyl of 12, 14, or 16 carbons in length, and 12-, 14-, 16-, or 18-BA:-acylbenzyldimethylammonium with acyl of 12, 14, 16, or 18 carbons in length.

neutral complexes between the (polyanionic) nucleic acid and the surfactant, leaving the hydrophobic tail of the surfactant facing the bulk phase[8]. These complexes are soluble in polar solvents, and they are dissociated by the addition of a salt. We reported that cationic surfactants could lyse cells, rendering their proteins soluble[9], and that the direct addition of cationic surfactants to cells could be used to isolate the RNA and DNA[10].

In attempting to apply this method to whole blood, we found that commercially available cationic surfactants were unable to lyse blood cells completely, and that they incompletely precipitated RNA from dilute solutions.

To overcome these inadequacies, we synthesized and tested several novel cationic surfactant solutions, finding (to our surprise) that the nature of the counter ion had a decisive influence on both blood lysis and RNA precipitation. Table 1*a* shows the size of the pellet obtained after centrifuging a mixture of blood and a series of test surfactants. Table 1*b* shows the ability of the surfactants to precipitate radioactive RNA from dilute solutions. Examination of these results shows that solutions prepared by neutralizing alkyltrimethylammonium hydroxides with dicarboxylic acids were capable both of lysing the blood well, and almost quantitatively precipitating the RNA.

Within limits, the length of the surfactant tail (the major determinant of the critical micellar concentration) had little influence on the ability of a cationic surfactant salt to precipitate RNA. This may be because (in concentrated aqueous mixtures), the surfactant exists in an equilibrium between one or more ordered structures in which the hydrophobic tails are juxtaposed (micelles, tubes, sheets, complexes with protein or with nucleic acids), and (as a minor component) surfactant monomer[11]. Increasing the length of the hydrophobic tail increases the stability of each of the ordered structures to the same extent, resulting in a decrease in the monomer concentration, but no change in the relative concentration of the ordered structures.

With the exception of the complex with nucleic acids, the cationic charge of the surfactant in the ordered structures is neutralized by the counter ion. Thus, unlike the tail length, the counter ion can have a marked differential impact on the relative stability of surfactant complex with nucleotide with respect to other ordered structures.

Further studies led us to select tetradecyltrimethylammonium oxalate (Catrimox-14) solution as a suitable reagent to isolate RNA from whole blood.

FIG. 1 Isolation of RNA from whole blood. Fifty or 100 µl blood anticoagulated with 1/10 vol. 3.2% sodium citrate was added to 1 ml 0.1 M Catrimox-14 surfactant and 0.1 M DTT and mixed. Fifteen minutes later, the mixture was centrifuged (16,000*g*, 5 min), and the supernatant was aspirated and discarded. The pellet was washed with 1 ml RNase free water, and dissolved in 100 µl 4 M guanidinium isothiocyanate, 0.2 M sodium acetate pH4 with occasional vortexing for 10 min. One hundred microlitres of phenol:chloroform:isoamyl alcohol pH 5.2; (25:24:1; Amresco, Solon Ohio) was then added and vortexed. After centrifugation (16,000 *g*, 2 min), the upper aqueous layer was added to 100 µl isopropanol, and the RNA was allowed to precipitate at −20 °C for 30 min. After centrifugation, the supernatant was discarded, the pellet was washed with 70% ice-cold ethanol, and dried briefly *in vacuo*. It was redissolved in a formaldehyde buffer and electrophoresed on a denaturing 1.2% agarose gel (65 × 100 mm), and stained with ethidium bromide. The figure is a photograph of the resulting gel under ultraviolet light. The size markers (stds: Gibco BRL, Gaithersburg, Maryland) are indicated in bases.

Details of the procedure

The blood sample (100 µl, fresh or anticoagulated with citrate) is mixed with the Catrimox-14 reagent, and transported to the laboratory. The RNA and DNA surfactant complexes are harvested by centrifugation, and the supernatant is carefully removed. The precipitated RNA can be recovered in one of two ways. In the first, the pellet is extracted with hot formamide containing a sodium acetate buffer which preferentially extracts the RNA, leaving most of the DNA as a loose pellet. The RNA is finally precipitated from the formamide by the addition of cold ethanol.

In the second method, the surfactant nucleotide pellet is extracted with 4.0 M guanidinium isothiocyanate, pH 4, which dissociates the complex by virtue of its ionic strength, and which also inhibits RNase. DNA, residual protein and other impurities are removed from the guanidinium solution by a single phenol-chloroform extraction, and the RNA is recovered by isopropanol precipitation. The formamide method has the advantage of avoiding the use of phenol. The yield of full length RNA is higher with the guanidinium method.

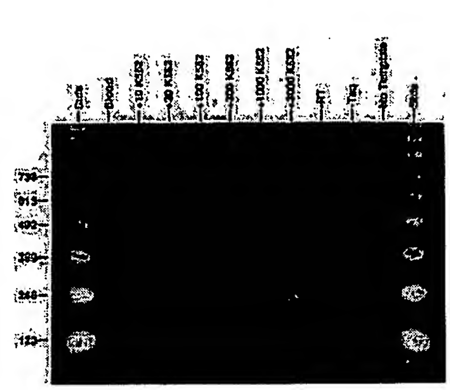


FIG. 2 RT-PCR amplification of *bcr/abl* oncogene. K562 human leukaemia cells (which express the *bcr/abl* hybrid oncogene) were mixed with blood, and (using precautions to prevent contamination[14]), the RNA was extracted as described in Fig. 1. The cDNA was made by incubating the RNA at 37 °C for 1 h with 200 units Maloney murine leukaemia virus reverse transcriptase (Gibco BRL) in 40 µl PCR buffer (50 mM KCl; 4 mM $MgCl_2$; 50 mM Tris, pH 8.4; 100 µg ml^{-1} bovine serum albumin) containing 20 units RNasin, 5 mM dithiothreitol, 20 pmol downstream primer ('CML-B' of Sawyer *et al.*[15]: 5'-TCAGACCCTGAGGCTCAAAGTC-3'), 1mM deoxynucleotide triphosphates (Pharmacia, Piscataway, New Jersey). The reaction was terminated by heating to 95 °C. At this temperature, 80 µl PCR buffer was added, with 20 pmol upstream primer ('C' of Delfau *et al.*[16]; 5'-GCTTCTCCCTGACATCCGTG-3') and 2.5 units of *Taq* DNA polymerase (AmpliTaq, Perkin Elmer-Cetus, Emeryville, California). The mixture was overlaid with mineral oil, and amplified by thirty cycles, each consisting of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min in a thermal cycler (Perkin Elmer). The PCR products were examined by ethidium bromide-stained agarose electrophoresis. The predicted amplimer of the *bcr/abl* mRNA cDNA is 219 bp. The lanes are (from left to right): standards (a 123-bp ladder, Gibco BRL); blood alone; blood plus 10, 30, 100, 300, 1,000, 3,000 K562 cells; blood plus 3,000 K562 cells, omitting reverse transcriptase; blood plus 300 K562 cells omitting *Taq* polymerase; amplification without a template; standards.

Figure 1 shows an ethidium bromide-stained agarose electrophoresis gel of RNA isolated from whole blood, using the Catrimox-14 surfactant and guanidinium method. The bands of rRNA are clearly seen with the usual smear of other species of RNA. Figure 2 shows the products of RT-PCR using primers that amplify a 219-base pair (bp) segment of the *bcr/abl* gene product in K562 leukaemic cells, which were mixed into normal blood. The expected amplimer was seen by ethidium fluores-

cence when as few as thirty K562 cells were added to the 100 µl blood sample, using only thirty cycles of amplification and a single pair of primers.

Discussion

The use of the cationic surfactant considerably simplifies the extraction of RNA from cells compared with conventional methods. The RNA-surfactant complex is not a substrate for RNase, so that once the blood is mixed with the surfactant, the specimen does not need to be processed immediately. As described above, the cationic surfactant method for whole blood calls for half the number of additions and one or two less centrifugations than the popular 'one-step' method, and it takes less than half the time to perform. RNA isolated from blood by the cationic surfactant method seems to be easy to amplify by RT-PCR, an important consideration because constituents (or their derivatives) of blood tend to inhibit PCR.

The need for a simple method for isolating RNA from whole blood in a form that can be amplified by PCR is great. Appropriate primers may enable the detection and quantitation of RNA encoded by several common pathogens including human immunodeficiency virus, parvovirus, hepatitis C, cytomegalovirus, mycobacteria, bacteria, and several tropical diseases. Leukocytes express immunoglobulins and several inflammatory mediators (including cytokines, adhesion receptors, and proteins related to blood coagulation) when stimulated, and assaying the corresponding mRNAs[12] may reveal patterns specific for certain autoimmune and other inflammatory diseases. An increasing number of leukaemias and lymphomas are known to have gene rearrangements giving rise to oncogenic mRNAs[13]. Figure 2 shows that a small number of cells with such a rearrangement can easily be detected in a blood sample using the method. Some genetic disorders, such as thalassaemia, may be easier to characterize by analysis of RNA in blood than by other means. Lastly, it is likely to be possible to perform HLA tissue typing and blood group typing by analysis of RNA in blood. □

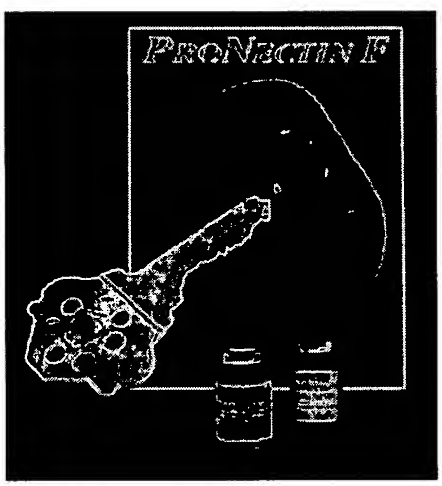
Donald E. Macfarlane and Christopher E. Dahle are in the Department of Internal Medicine, the University of Iowa, Iowa City, Iowa 52242, USA. DEM is also president of Iowa Biotechnology Corporation, Technology Innovation Center, the University of Iowa, Iowa City, Iowa 52242, USA. *This work was supported by a grant from Iowa Biotechnology Corporation.* *For more information, fill in reader service number 100.*

1. Kawasaki, E. S. in *PCR Protocols: A Guide to Methods and Applications* (eds Innis, M. A., Gelfaud, D. H., Sninsky, J. J. & White, T. J.) 21–38 (Academic, New York, 1990).
2. Chromczynski, P. & Sacchi, N. in *Current Protocols in Molecular Biology* Unit 4.2, Suppl. 14 (Wiley, New York, 1991).
3. Higuchi, R. in *PCR Technology* (ed. Erlich, H. A.) (Stockton, New York, 1989).
4. Reddi, K. K. & Holland, J. F. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2308–2310 (1976).
5. Jones, A. S. *Biochim. biophys. Acta* **10**, 607–612 (1953).
6. Aubel-Sadron, G., Beck, G. & Ebel, J. -P *Biochim. biophys. Acta* **53**, 11–18 (1961).
7. Ralph, R. K. & Bellamy, A. R. *Biochim. biophys. Acta* **87**, 9–16 (1964).
8. Hayakawa, K. & Kwak, J. C. T. in *Cationic Surfactants, Physical Chemistry* (eds Rubingh, D. N. & Holland, P. M.) (Marcel Dekker, New York, 1991).
9. Macfarlane, D. E. *Analyt. Biochem.* **176**, 457–463 (1989).
10. Macfarlane, D. E. US Patent #5,010,183 (1991).
11. Reck, R. A. *Encyclopedia of Chemical Technology* **19**, 521–531 (1982).
12. Dallam, M. J., Montgomery, R. A., Larsen, P. L., Wanders, A. & Wells, A. F. *Immunol. Rev.* **119**, 163–179 (1991).
13. Nichols, J. & Nimer, S. D. *Blood* **80**, 2953–2963 (1992).
14. Kwok, S. Perkin Elmer Biotechnology Catalogue, Norwalk, Connecticut (1992).
15. Sawyers, C. L., Timson, L., Kawasaki, E. S., Clark, S. S., Witte, O. N. & Champlin, R. *Proc. natn. Acad. Sci. U.S.A.* **87**, 563–567 (1990).
16. Delfau, M. -H., Kerckaert, J. -P, dHooge, M. C., Fenaux, P., Laï, J- L., Jouet, J. -P. & Grandchamp, B. *Leukemia* **4**, 1–5 (1990).

Trends in tissue culture

An agent to eliminate mycoplasma from cultures, cell attachment factors, media, bioreactors, incubators, filters and a selection of cell and tissue culture catalogues are featured.

ICN Flow announces the availability of MRA, a **mycoplasma removal agent** that ICN developed in conjunction with Dianippon Pharmaceutical of Japan *(Reader Service No. 101)*. MRA, a water-soluble oxoquinoline-carboxylic acid derivative, is designed to remove contamination from plant and animal cell cultures and is intended to be useful in research laboratories where valuable cell lines may be at risk. Contaminated cells are cultured with MRA for a week. After removal of the agent, ICN says that the cells will not suffer a recurrent infection with the original mycoplasma. The same agent can also be used as a preventative measure where there may be a danger of mycoplasma contamination in serum or trypsin. The agent is supplied in a 5-ml vial. ICN says that the 50 mg ml^{-1} solution is sufficient for decontaminating up to 25 cell cultures.



ProNectin F cell attachment factor.

ProNectin F from Protein Polymer Technologies is a genetically engineered **cell attachment factor** that is designed to facilitate the transfer of many anchorage-dependent cell lines from serum-containing to serum-free media *(Reader Service No. 102)*. The company says that use of ProNectin eliminates the need for the laborious 'hit or miss' serum weaning protocols that are usually required to adapt cells to low serum, serum-free or protein-free media.

The PAP pen from Research Products International is a special **marking pen** designed for drawing thin film-like barriers on microscope slides *(Reader Service No. 103)*. This barrier creates the proper surface tension needed to hold antibody solution and other specimens within a target area. The pen contains a special water-repellent formulation that is insoluble in alcohol and acetone, and is effective for immunoperoxidase staining, frozen section and fluorescent antibody staining methods. Two models are available: one with a 4-mm tapered point for over 800 applications, and a Mini PAP pen with a 2.5-mm tip for over 400 applications.

A new indicator dye that reacts in response to cellular metabolism and a microplate fluorometer make up the new **cell proliferation assay system** from Alamar *(Reader Service No. 104)*. The indicator dye, alamarBlue, produces both a fluorometric and a colorimetric response to cellular proliferation in mammalian cells, bacteria and fungi. It can also be used to establish the relative cytotoxicity of agents

L

ANALYTICAL BIOCHEMISTRY 162, 156–159 (1987)

Single-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate–Phenol–Chloroform Extraction

PIOTR CHOMCZYNSKI*,[1] AND NICOLETTA SACCHI†

*Laboratory of Biochemistry and Metabolism, NIDDK, National Institutes of Health, Bethesda, Maryland 20892, and †Laboratory of Molecular Oncology, NCI, National Institutes of Health, Frederick, Maryland 21701

Received May 27, 1986

A new method of total RNA isolation by a single extraction with an acid guanidinium thiocyanate–phenol–chloroform mixture is described. The method provides a pure preparation of undegraded RNA in high yield and can be completed within 4 h. It is particularly useful for processing large numbers of samples and for isolation of RNA from minute quantities of cells or tissue samples. © 1987 Academic Press, Inc.

KEY WORDS: nucleic acids; RNA; messenger RNA; purification; gene expression; human cells.

Guanidinium thiocyanate and chloride are among the most effective protein denaturants (1,2). As a strong inhibitor of ribonucleases, guanidinium chloride was first introduced as a deproteinization agent for isolation of RNA by Cox (3). Since then guanidinium extraction has become the method of choice for RNA purification, replacing phenol extraction. Guanidinium methods have been used successfully by Chirgwin *et al.* (4) to isolate undegraded RNA from ribonuclease-rich tissues like pancreas. Chirgwin's protocol for ultracentrifugation of a guanidinium thiocyanate lysate through a CsCl cushion has become one of the most frequently used for isolation of undegraded RNA. In the present report, a new rapid procedure combining guanidinium thiocyanate and phenol–chloroform extraction is described. A combination of guanidinium and hot phenol for RNA isolation has been reported by Feramisco *et al.* (5). The method we describe differs in that it converts the guanidinium–hot phenol method to a single-step extraction which allows isolation of RNA in 4 h and provides both high yield and purity of undegraded RNA preparations. By eliminating the ultracentrifugation step of the guanidinium–CsCl method this procedure allows the simultaneous processing of a large number of samples. In addition, this new procedure permits recovery of total RNA from small quantities of tissue or cells making it suitable for gene expression studies for which only a limited quantity of biological material is available.

MATERIALS AND METHODS

Reagents. The denaturing solution (solution D) was 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1 M 2-mercaptoethanol. To minimize handling of guanidinium thiocyanate (hazardous) a stock solution was prepared as follows: 250 g guanidinium thiocyanate (Fluka) was dissolved in the manufacturer's bottle (without weighing) with 293 ml water, 17.6 ml 0.75 M sodium citrate, pH 7, and 26.4 ml 10% sarcosyl at 65°C. This stock solution can be stored at least 3 months at room temperature. Solution D was prepared by adding 0.36 ml 2-mercaptoethanol/50 ml of stock

[1] To whom correspondence should be addressed at University of Cincinnati College of Medicine, 231 Bethesda Ave., Rm. 5564, Cincinnati, OH 45267-0547.

0003-2697/87 $3.00

solution. This solution can be stored 1 month at room temperature.

Phenol (nucleic acid grade, Bethesda Research Laboratory) saturated with water was kept at 4°C up to 1 month.

Small-scale RNA preparations were carried out in 4- or 15-ml disposable polypropylene tubes (Falcon, Cat. Nos. 2063, 2059).

Experimental procedure. The acid guanidinium – phenol – chloroform (AGPC)[2] method was used to isolate RNA from both tissues and cultured cells. The following protocol describes isolation of RNA from 100 mg of rat mammary tissue.

Immediately after removal from the animal, the tissue was minced on ice and homogenized (at room temperature) with 1 ml of solution D in a glass–Teflon homogenizer and subsequently transferred to a 4-ml polypropylene tube. Sequentially, 0.1 ml of 2 M sodium acetate, pH 4, 1 ml of phenol (water saturated), and 0.2 ml of chloroform–isoamyl alcohol mixture (49:1) were added to the homogenate, with thorough mixing by inversion after the addition of each reagent. The final suspension was shaken vigorously for 10 s and cooled on ice for 15 min. Samples were centrifuged at 10,000*g* for 20 min at 4°C. After centrifugation, RNA was present in the aqueous phase whereas DNA and proteins were present in the interphase and phenol phase. The aqueous phase was transferred to a fresh tube, mixed with 1 ml of isopropanol, and then placed at −20°C for at least 1 h to precipitate RNA. Sedimentation at 10,000*g* for 20 min was again performed and the resulting RNA pellet was dissolved in 0.3 ml of solution D, transferred into a 1.5-ml Eppendorf tube, and precipitated with 1 vol of isopropanol at −20°C for 1 h. After centrifugation in an Eppendorf centrifuge for 10 min at 4°C the RNA pellet was resuspended in 75% ethanol, sedimented, vacuum dried (15 min), and dissolved in 50 μl 0.5% SDS at 65°C for 10 min. At this point the RNA preparation could be used for poly(A)$^+$ selection by oligo(dT) chromatography, Northern blot analysis, and dot blot hybridization. Isopropanol precipitation can be replaced by precipitation with a double volume of ethanol.

The protocol for RNA isolation by the AGPC method is outlined in Table 1. Until the last step, ribonuclease is inhibited by the presence of 4 M guanidinium. Therefore no additional precaution is required to protect RNA from degradation. The final RNA preparation can be dissolved in water or in 1 mM EDTA, pH 8, solution, treated with diethyl pyrocarbonate (DEP) (6). We recommend, however, to use 0.5% SDS (DEP treated) which is a weak inhibitor of ribonuclease and may diminish the effect of accidental contaminations during the further use and storage of RNA samples.

The AGPC method was used for both small scale (3 mg tissue or 10^6 cells) and large scale (30 g tissue) RNA preparations. The method was also used to isolate RNA from cultured cell lines: human and mouse hematopoietic cell lines, breast MCF-7 cells, human normal lymphocytes, and bone marrow blasts, and rat thyroid FRTL-5 cells. RNA could be isolated from cells grown in suspension (100 μl of solution D per 10^6 cells) or in monolayer. Cells grown in monolayer were lysed directly in the tissue culture dish by the addition of denaturing solution (1.8 ml of solution D for 10-cm-diameter tissue culture dish).

TABLE 1

AGPC PROTOCOL FOR RNA ISOLATION

1. Extraction	Solution D, 0.2 M sodium acetate, pH 4, phenol, chloroform (1:0.1:1:0.2)
2. Precipitation	1 vol isopropanol
3. Reprecipitation	Solution D, 1 vol isopropanol
4. Wash	75% ethanol
5. Solubilization	0.5% SDS

[2] Abbreviations used: AGPC, acid guanidinium thiocyanate–phenol–chloroform; SDS, sodium dodecyl sulfate; DEP, diethyl pyrocarbonate.

TABLE 2

COMPARISON OF PARAMETERS OF RNA PREPARATIONS OBTAINED BY THE AGPC METHOD AND BY THE GUANIDINIUM–CsCl METHOD[a]

Method	Ratio[b] (260/280)	DNA[c]	Yield[d] (μg RNA/mg tissue)	25K casein mRNA[e] (cpm/μg RNA)
AGPC	1.85 ± 0.04	ND	1.76 ± 0.03	2632 ± 143
Guanidinium–CsCl	1.75 ± 0.05	ND	1.52 ± 0.03	2597 ± 127

[a] Results represent averages of duplicate analyses of three RNA preparations. ND = not detectable.
[b] Absorption of RNA preparations was determined at 260 and 280 nm.
[c] DNA was quantitated by Burton's reaction (7).
[d] Yield was calculated assuming $A_{1\,cm}$/0.1% at 260 = 25.
[e] 25K casein mRNA was quantitated by hybridization with a ^{32}P-labeled 25K casein cDNA probe (10).

RESULTS AND DISCUSSION

RNA was prepared from rat mammary tissue by the AGPC extraction and compared with RNA isolated in parallel by ultracentrifugation of guanidinium lysate through a CsCl cushion (4). Data in Table 2 show that RNA isolated by the AGPC method contained less contaminating proteins, as judged by the 260/280 ratio, than RNA isolated by the guanidinium–CsCl method. An additional wash with 75% ethanol of the RNA pellet during the AGPC extraction increased to about 2 the 260/280 ratio of the isolated RNA preparation. DNA was undetectable in both preparations using Burton's method and both had a similar content of 25K casein mRNA. The AGPC extraction, however, resulted in a slightly higher yield of RNA.

Agarose-gel resolution patterns of total RNA isolated from rat mammary gland and rat liver by both the AGPC and the guanidinium–CsCl methods are shown in Fig. 1. Both preparations reveal similar patterns, but liver RNA isolated by the AGPC method contained a greater amount of low-molecular-weight RNA (4–5 S).

The AGPC procedure was used for expression studies of the ets-2 gene (9) in human bone marrow myeloblasts (low-abundancy mRNA), the 25K casein gene (10) in rat mammary gland, and the thyroglobulin gene (11) in FRTL-5 cells (high-abundancy mRNAs). AGPC extracted RNA from human myeloblasts was subjected to oligo(dT) chromatography, and the poly(A)$^+$ fraction was analyzed for ets-2 mRNA content by Northern blot analysis (Fig. 2A). Three transcripts of 4.7, 3.4, and 2.8 kb were detected by an ets-2 human genomic probe (9) as previously observed using the guanidinium–CsCl method. Total RNA isolated from mammary explants cultured with a lactogenic hormone combination (insulin, prolactin, and hydrocortisone) were probed with 25K casein cDNA and β-actin cDNA (12)

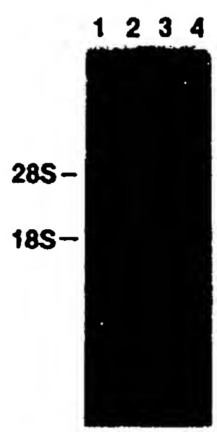


FIG. 1. Electrophoresis of RNA isolated by the AGPC method (lanes 1, 3) and by the guanidinium–CsCl method (lanes 2, 4) from rat mammary gland (lanes 1, 2) and rat liver (lanes 3, 4). RNA preparations (3 μg) were electrophoresed in formaldehyde–agarose (1%) minigel (8). The gel was washed in water two times for 30 min and stained with ethidium bromide.

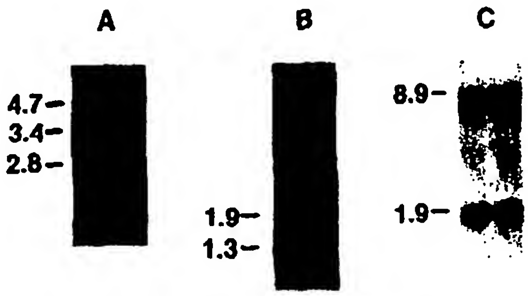


FIG. 2. Northern blot analysis of RNA isolated by the AGPC method. (A) Total RNA from human myeloblasts was chromatographed on oligo(dT) column (15) and 2 μg of the poly(A)$^+$ fraction was analyzed for ets-2 mRNAs (4.7, 3.4, and 2.8 kb); (B) 1.5 μg of total RNA from rat mammary explants was analyzed for 25K casein mRNA (1.3 kb) and β-actin mRNA (1.9 kb); (C) 1.6 μg of total RNA from rat thyroid FRTL-5 cells was analyzed for thyroglobulin mRNA (8.9 kb) and β-actin mRNA. RNA preparations were electrophoresed, transferred to Gene Screen Plus membrane, and autoradiographed as described (9,12).

(Fig. 2B). A 1.3-kb casein mRNA and 1.9-kb β-actin mRNA were detected. Both messengers migrated as distinct bands with no signs of degradation. Thus, despite a high level of ribonuclease activities in rat mammary tissue (13,14), the AGPC method assured high quality of isolated mammary RNA. Finally, undegraded high-molecular-weight thyroglobulin mRNA (8.9 kb) could be visualized in total RNA from rat FRTL-5 cells using a rat thyroglobulin cDNA probe (11) (Fig. 2C).

Presented results show that the AGPC method is a useful alternative to the previously described methods of RNA isolation. The AGPC extraction provides high yield and the extracted RNA is both pure and undegraded. Due to its simplicity and the elimination of ultracentrifugation, the AGPC method allows simultaneous processing of a large number of samples. The method proved to be particularly useful for RNA isolation from as few as 10^6 cells or 3 mg of tissue (human pituitary tumor). The degradation and loss of RNA is minimized by the limited handling involved in this technique. The AGPC method may therefore be useful for clinical investigations that employ gene expression such as protooncogene expression as a molecular marker of malignancy and tumor progression.

REFERENCES

1. Nozaki, Y., and Tanford, C. (1970) *J. Biol. Chem.* **245,** 1648–1652.
2. Gordon, J. A. (1972) *Biochemistry* **11,** 1862–1870.
3. Cox, R. A. (1968) *in* Methods in Enzymology (Grossman, L., and Moldave, K., Eds.), Vol. 12, Part B, pp. 120–129, Academic Press, Orlando, FL.
4. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
5. Feramisco, J. R., Helfman, D. M., Smart, J. E., Burridge, K., and Thomas, G. P. (1982) *in* Molecular Cloning (Maniatis, T., Fritsch, E. F., and Sambrook, J., Eds.), pp. 194–195, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
6. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *in* Molecular Cloning (Maniatis, T., Fritsch, E. F., and Sambrook, J., Eds.), p. 190, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
7. Burton, K. (1956) *Biochem. J.* **62,** 315–322.
8. Kopchik, J. J., Cullen, B. R., and Stacey, D. W. (1981) *Anal. Biochem.* **115,** 419–423.
9. Sacchi, N., Watson, D. K., Geurts vanKessel, A., Hagemeijer, A., Kersey, L., Drabkin, H., Patterson, D., and Papas, T. S. (1986) *Science* **231,** 379–381.
10. Chomczynski, P., Qasba, P., and Topper, Y. J. (1984) *Science* **226,** 1326–1328.
11. Musti, A. M., Avvedimento, E. V., Polistina, C., Ursini, V. M., Obici, S., Nitsch, L., Cocozza, S., and DiLauro, R. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 323–327.
12. Chomczynski, P., Qasba, P., and Topper, Y. J. (1986) *Biochem. Biophys. Res. Commun.* **134,** 812–818.
13. Liu, D. K., Liao, W. S., and Fritz, P. J. (1977) *Biochemistry* **16,** 3361–3368.
14. Liu, D. K., Kulick, D., and Williams, G. (1979) *Biochem. J.* **178,** 241–244.
15. Aviv, H., and Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 1408–1412.

M

Molecular and Cellular Probes (2002) **16**, 25–30
doi:10.1006/mcpr.2001.0392, available online at http://www.idealibrary.com on IDEAL

Quantitative analysis of beta-actin, beta-2-microglobulin and porphobilinogen deaminase mRNA and their comparison as control transcripts for RT-PCR

J. Lupberger, K.-A. Kreuzer, G. Baskaynak, U. R. Peters, P. le Coutre and C. A. Schmidt*

Department of Medicine, Division of Hematology/Oncology, Charité Virchow-Klinikum, Humboldt University Berlin, Augustenburger Platz 1, Berlin, FRG

(Received 2 July 2001; accepted for publication 24 September 2001)

Quantitation of target mRNAs using the reverse-transcription polymerase chain reaction found a widespread field of application in diverse biomedical diagnostic assays. However, the problem of varying sample quality has to be solved by correcting target molecule amounts through detection of an endogenous control template. The choice of an appropriate reference gene is still object of debate as pseudogene co-amplification and expression level variations may limit the usefulness of some currently used reference reactions. We compared quantitative expression levels of the commonly used endogenous reference genes beta-actin (β-actin), beta-2-microglobulin (β_2-MG) and porphobilinogen deaminase (PBDG) using the TaqMan chemistry. With these assays we investigated the respective expression patterns in K562 cells and leucocytes of normal individuals as well as of malignoma patients. In K562 cells $1544+246$ β-actin, 65 ± 30 β_2-MG and 22 ± 8 PBDG copies/cell were detected. In normal leucocytes 491 ± 97 β-actin, 40 ± 17 β_2-MG and <1 PBDG copies/cell were quantified. Leucocytes of various malignancies exhibited 84 ± 51 β-actin, 106 ± 8 β_2-MG and <1 PBDG copies/cell. We conclude that β_2-MG is the most suitable reference gene tested as its variation between different sample origins and within distinct cell types was acceptable low.

KEYWORDS: reverse transcription polymerase chain reaction, controls, reference genes, beta-actin, beta-2-microglobulin, porphobilinogen-deaminase.

INTRODUCTION

Real-time fluorescence PCR provides a highly sensitive, reproducible and fast way for the quantitation of nucleic acid sequences. Since it was first described in 1995 an increasing number of assays using this technique are going to replace previous quantitative PCR methods in routine laboratories.

Its basic principle is the usage of a non-extendable fluorescent probe, which is complementary to a specific sequence within the target sequence. On its terminal nucleotides it is labelled with different fluorescent dyes. The quencher dye, which is typically covalently attached to the 3′-terminus suppresses fluorescent emission of the reporter dye, located at the 5′-terminus. This phenomenon is called the 'Fluorescence Resonance Energy Transfer' (FRET)[1,2] and is based on the close proximity of both dyes resulting in a low measurable fluorescent signal.

* Author to whom all correspondence should be addressed: Priv.-Doz. Dr. med. C.A. Schmidt, Department of Medicine, Division of Haematology/Oncology, Charité Virchow-Klinikum, Humboldt University Berlin, Augustenburger Platz 1, 13353 Berlin, FRG, Tel. +49 (030) 450-553663; Fax. +49 (030) 450-559929; E-mail: christian.schmidt@charite.de

0890–8508/02/010025+06 $35.00/0

In case of hybridization to the target sequence the Taq DNA polymerase cleaves the probe from the strand during extension of the primers via its 5′-3′ exonuclease activity.[3] Thus, the distance between both dyes increases and the reporter dye starts to emit a detectable fluorescent signal whose accumulation is proportional to the starting amount of cDNA in the reaction. Compared to former semi-quantitative approaches the advantage of such real time PCR techniques is the detection at the log-phase of the amplification where no limiting reaction factors might influence the reaction kinetics.

In general, a major problem of cDNA synthesis is the sensitivity and potential degradation of RNA during the entire sample processing. Thus, varying sample qualities need to be corrected by co-amplification of an internal standard. The commonly used endogenous references belong to the group of 'housekeeping genes'. In an ideal setting these genes are constitutionally expressed by all cell types and should not be affected by any human disease. But even widely used reference genes like beta-actin, glyceraldehyde-3-phosphate or hypoxanthine phosphoribosyltransferase (HPRT)[4] were criticised in the past because of possible pseudogene co-amplifications. Furthermore, actual transcription rates of most reference RNA transcripts with respect to different sampling procedures and various diseases are still unknown.

In order to evaluate which reference genes may be suitable for control reactions using the so called TaqMan chemistry we developed absolute quantitative RT-PCR assays for beta-actin (β-actin), beta-2-microglobulin (β_2-MG) and porphobilinogen deaminase (PBGD). Furthermore, in order to study their expression patterns *in vitro* and *in vivo* we determined their amount in K562 cells, normal peripheral blood cells and peripheral leucocytes of malignoma patients.

MATERIALS AND METHODS

The following samples were investigated: separate RNA extractions of K562 cells ($n=4$), normal leucocyte preparations ($n=4$) as well as peripheral blood leucocytes of chronic myelogenous leukaemia ($n=1$), chronic lymphocytic leukaemia ($n=1$), acute myelogenous leukaemia ($n=1$), mamma carcinoma ($n=1$), B-cell non-Hodgkin lymphoma ($n=2$), multiple myeloma ($n=2$) and colon carcinoma ($n=2$). The K562 cell line grew in 90% RPMI 1640 medium including glutamax-I and 10% fetal bovine serum (Life Technologies, Karlsruhe, Germany). To minimize cell cycle variations samples were all taken during the log phase of growth. All extractions were performed immediately after collection with a maximum processing time of three hours until storage of extracted RNA (−80°C). Peripheral blood samples were collected into sterile heparin tubes (Sarsted, Numbrecht, Germany). After centrifugation for 5′ at $400 \times g$ all leukocytes from the blood pellet were separated by selective erythrocyte lysis (QIAmp RNA Blood Mini Kit; Qiagen, Hilden, Germany). Pellets were resuspended in phosphate buffered saline (PAA Laboratories, Linz, Austria) and filtrated through a 40 µm cell strainer (Becton Dickinson, Franklin Lakes, USA) in order to remove larger cell precipitations after the resuspension of the pellet. Cell density was subsequently assessed using an automated cell counter (Casy 1; Schärfe Systems, Reutlingen, Germany) and the sample were diluted to a cell concentration of $2–4 \times 10^5$ per ml. With regard to potential deviations in terms of different cell densities we performed RNA extractions of one cell sample at three different cell dilutions in parallel. Thus, 9 different samples were obtained from one origin: 3×10^6, 3×10^5 and 3×10^4 cells (QIAmp RNA Blood Mini Kit; Qiagen, Hilden, Germany) and suspended in 30 µl Tris/HCl buffer. A maximum amount of 1000 ng cell line RNA and 8 µl of blood RNA were reverse transcribed (RT) into cDNA. The RT mixture contained 100 µM random hexamer primers (pd(N)$_6$; Amersham Pharmacia Biotech, San Francisco, USA), 2·5 mM dNTP (Life Technologies, Karlsruhe, Germany), 10 × PCR buffer (Life Technologies, Karlsruhe, Germany), 0·1 M dithiotreitol (DTT; Life Technologies, Karlsruhe, Germany). After an initial denaturation ribonuclease inhibitor (recombinant RNasin; Promega, Madison, USA) and RNA polymerase (SuperScript RT; Life Technologies, Karlsruhe, Germany) was added. cDNA was stored at −20°C until being assayed.

We performed TaqMan PCR with a single pair of primers for every reference gene separately. For β-actin primer oligonucleotides were designed to hybridize within the 5′-untranslated region of exon 1 (sense) and within exon 2 (antisense) of human β-actin cDNA.[5] The sense primer of β_2-MG was within exon 1 and 2 (5′-TACATgTCTCgATCCCACTTAACTAT) and the antisense primer within exon 2 and 3 (5′-AgCgTACTCCAAAgATTCAggTT) of human β_2-MG cDNA. The intron spanned primer sequences prevented genomic DNA co-amplification.

For β_2MG we used the following fluorescent probe oligonucleotide: 5′-CTCACgTCATCCAgCAgAgAATggAAAgTCA, which was labelled at the 5′-terminus with the reporter dye 6-carboxy-fluorescein phosphoramidite (FAM) and 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3′-terminus as the quenching dye. The sense primer for PBGD was located within

exon 1 and 2 (5'-TgCAACggCggAAgAAAACA), and the antisense primer in exon 7 (5'-AgATggCTC-CgATgg) of human PGBD cDNA. Here the probe sequence was 5'-CCAAAgATgAgAgTgATTCgCgTg, also labelled with the reporter dye FAM (5'- terminus) and the quenching dye TAMRA (3'-terminus). All oligonucleotides were synthesised by TIB Molbiol Inc (Berlin, Germany).

For TaqMan PCR calibration we generated a calibrator dilution series for each reference gene ranging from 10^7 to 10 copies per 2 µl. The required amount of specific cDNA was amplified from RT material of healthy human blood with specific primers. In case of β_2-MG we performed plasmid specific PCR after cloning the target sequence into the pCR2.1-TOPO vector (TOPO TA Cloning Kit; Invitrogen Corp., Carlsbad, USA) using the M13 priming site on the LacZα fragment. After extraction from a 3% agarose gel (QIAquick Gel Extraction Kit; Qiagen, Hilden, Germany) the optical density was measured at 260 nM in a photometer and the number of copies was calculated. On every TaqMan PCR plate all the calibration standards and cDNA samples were detected in duplicates using the ABI PRISM 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, USA) over 45 PCR cycles.

RESULTS

During TaqMan PCR fluorescence kinetic of a given sample follows a sigmoid growth function (Fig. 1A). The cycle when the signal exceeds a set threshold value (Ct; threshold cycle) is directly correlated to the calculated copy value of these dilution. Thus, the absolute copy amount of an unknown DNA sample can be calculated from the regression curve of the logarithmic scaled standard Ct values against the linear scaled standard copy amounts (Fig. 1B). With our approach we obtained correlation coefficients (r) for beta-actin of 0.999, for β_2-MG of 0.999 and for PBGD of 0.998. Figure 1C shows the corresponding results of the used standard series on a 3% agarose gel. All assays were able to amplify a minimum amount of 10 copies target cDNA per 2 µl sample. Intra-assay coefficients of variation (CV) were 6% (β-actin), 10% (β_2-MG) and 27% (PBGD). Inter-assay CV were 7% (β-actin), 3% (β_2-MG) and 16% (PBGD), as calculated by variation from the mean of three separate measurements of the same cDNA. Genomic DNA samples remained steadily negative over 45 cycles of TaqMan PCR. Absolute copy numbers were calculated back to the initial cell numbers, which were set into the RNA extraction. As a result we obtained a copy/cell-ratio representing the average target RNA amount per cell. In Figure 2 this ratio is shown obtained from the different cell types. The average transcript level of beta-actin in the K562 extractions was 1544 copies/cell (SD: 246 copies/cell), 491 copies/cell (SD: 97 copies/cell) in the healthy blood donors and 84 copies/cell (SD: 51 copies/cell) in leucocytes derived from different cancer patients was. Differences between the cell types were significant as assessed by Students' t-test ($P<0{\cdot}001$). The average transcript amount of β_2-MG in K562 was 65 copies/cell (SD: 30 copies/cell), 40 copies/cell (SD: 17 copies/cell) in normal leucocytes and 106 copies/cell (SD: 8 copies/cell) in cancer patients (P-values between each group was >0·05 but <0·001 between K562 cells and the cancer patients; n.s.). For PBGD, the average expression level of K562 was 22 copies/cell (SD: 8 copies/cell), 0·007 copies/cell (SD: 0·003 copies/cell) in healthy blood donors leucocytes and 0·001 copies/cell (SD: 0·0009 copies/cell) in leucocytes of the cancer patients. The P-value between healthy blood donors and cancer patients was >0·001 (n.s.), while means were significantly different between K562 cells, normal leucocytes and cancer patient leucocytes ($P<0{\cdot}001$).

DISCUSSION AND CONCLUSIONS

The advantages of the TaqMan method regarding absolute quantification of DNA sequences can also be used for cDNA samples if appropriate control reactions are performed. However, this can be complicated by altered reference gene transcription within one individual or between different sample material.[6] In search of a suitable control target we investigated three widely used reference genes with respect to their expression stability. We found comparable transcription levels within and between all samples for β_2-MG and conclude that it is the most suitable endogenous reference from those tested. PBGD although shows a comparable average transcription rate in healthy blood donors and different cancer patients, but in the latter case with relatively high variations and with a very low absolute expression (<1 copies/cell). The differences of β-actin in all tested cells are acceptable, but, surprisingly, the degree of its expression varies from K562 cells > cancer patients > normal donors.

Furthermore, β_2-MG appears to be expressed in a suitable amount per cell. As one single PBGD RNA transcript does represent less than one cell it can be speculated that it does not fulfil the criteria of an 'housekeeping gene' which is expected to be expressed by every single cell. Possibly, PBGD transcription is restricted to certain subpopulations of

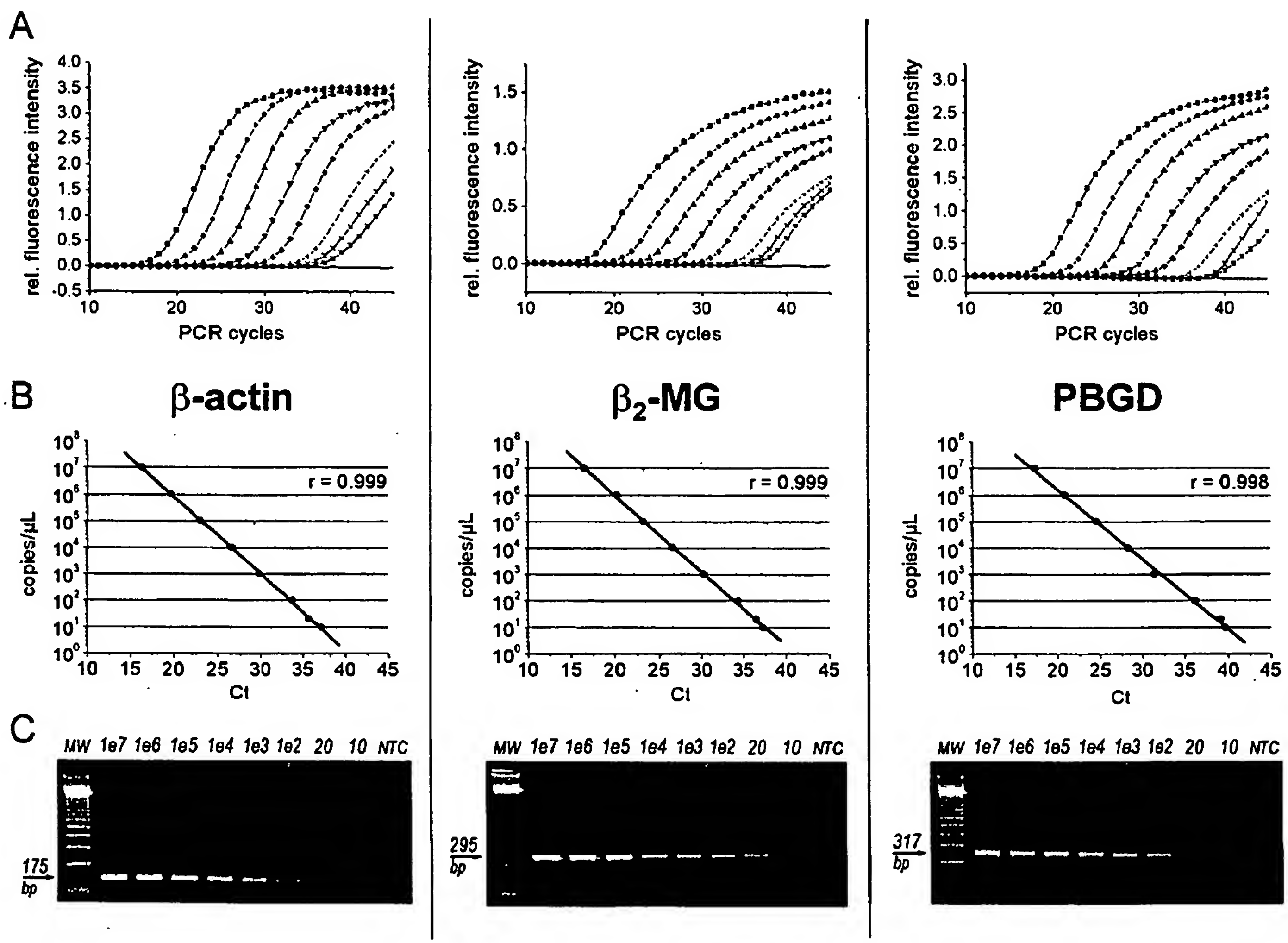


Fig. 1. Quantitative TaqMan PCR of the calibrator preparations of β-actin (left), β_2-MG (middle) and PBGD (right). (A) Increasing fluorescence intensity during cycling. (B) Calibrator curves obtained by correlation of the Ct values (threshold cycles) and initial calibrator concentrations. (C) Conventional PCR results of calibrators by agarose gel electrophoresis and ethidium bromide staining. MW, 123 bp molecular weight marker. NTC, no template control.

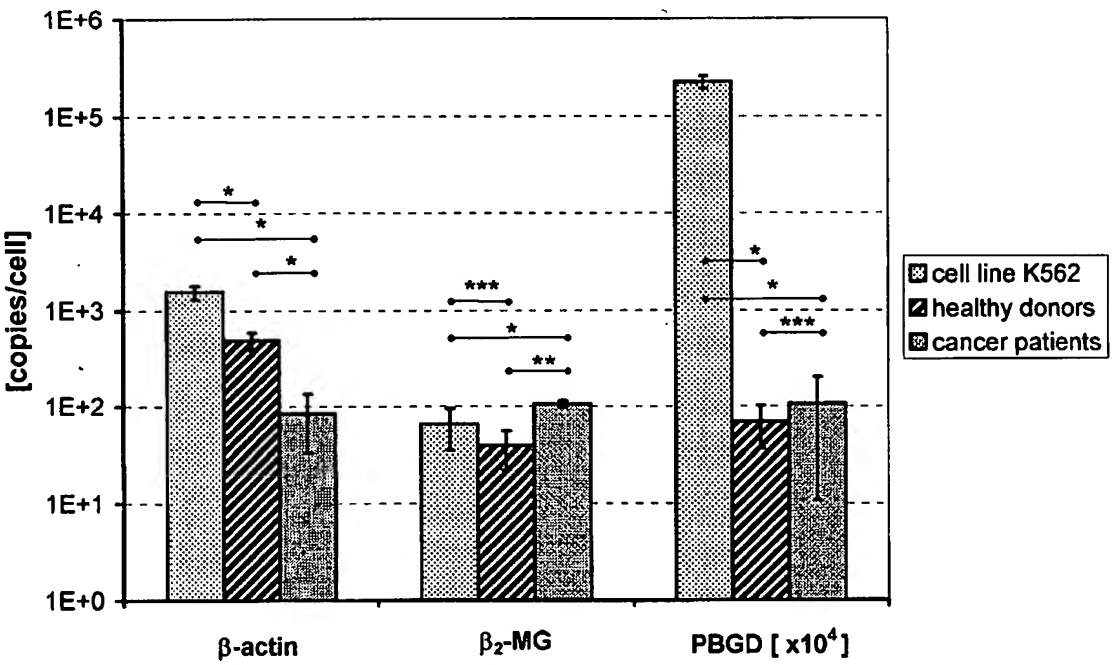


Fig. 2. Expression of reference genes in K562 cells, leucocytes of healthy blood donors and leucocytes of various cancer patients. For graphical reasons absolute amounts of PBGD transcripts are multiplicated by 10^4. *P*-values of significance: *<0·001; **<0·01; ***>0·05.

leucocytes. Finally, we were able to detect β-actin and β_2-MG transcripts in as little as 10 000 cells. Even if not representative for a sufficient number of cells these genes can be used to detect the blank existence of a minimum of 10^4 cell/per reaction.

It was claimed that RT-PCR has a sensitivity of 1 molecule per 10^6 cells. However, this is an theoretical assumption as the RNA content of 10^6 cells (5–8 μg)[7] cannot be analyzed in a single reaction. Thus, an amount of cDNA must be defined which represents a minimum number of cells. According to our observation 10^5 cells harbour $4{\cdot}9 \times 10^7$ β-actin copies or $3{\cdot}9 \times 10^6$ β_2-MG copies (healthy blood donors). We therefore suggest that these molecule numbers should be considered to guarantee that the quantitative results represent at least 10^5 cells.

It is now of interest whether all leucocyte types contribute equally to the absolute amount of reference genes which again is obligatory for constitutionally expressed control genes. Furthermore, their contribution within and between different tissues is of importance for future investigations. Further studies should aim to clarify this and possibly to identify superior control RNAs.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Deutsche José Carreras Leukämie-Stiftung (DJCLS-R22).

REFERENCES

1. Livak, K. F., Flood, S. J., Marmaro, J., Giusti, W. & Deetz, K. (1995). Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods and Applications* **6**, 357–62.
2. Förster, V. T. (1948). Zwischenmolekulare Energiewanderung und Fluoreszenz. *Annals of Physics* **2**, 55–75.
3. Holland, P. A., Abramson, R. D., Watson, R. & Gelfand, D. H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5′-3′ exonuclease activity of Thermus aquaticus DNA polymerase. *Proceedings of the National Academy of Science of the United States of America* **88**, 7276–80.
4. Sellner, L. T. & Turbett, G. R. (1996). The presence of a pseudogene may affect the use of HPRT as an endogenous mRNA control in RT-PCR. *Molecular and Cellular Probe* **10**, 481–3.

5. Kreuzer, K. A., Lass, U., Landt, O., Nitsche, A., Laser, J., Ellerbrok, H. *et al.* (1999). Highly sensitive and specific fluorescence reverse transcription-PCR assay for the pseudogene-free detection of beta-actin transcripts as quantitative reference. *Clinical Chemistry* **45**, 297–300.
6. Wu, Y. Y. & Rees, J. L. (2000). Variation in epidermal housekeeping gene expression in different pathological states. *Acta Dermato-Venereologica* **80**, 2–3.
7. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. *et al.Current Protocols in Molecular Biology,* Suppl. 36. Harvard Medical School: John Wiley & Sons: 4.2.9.

N

Promega

Technical Bulletin

RNAgents® Total RNA Isolation System

INSTRUCTIONS FOR USE OF PRODUCTS Z5110 AND Z5651.

www.promega.com

PRINTED IN USA.
Revised 3/05

AF9TB087 0305TB087

Part# TB087

Promega

RNAgents® Total RNA Isolation System

All technical literature is available on the Internet at www.promega.com/tbs
Please visit the web site to verify that you are using the most current version of this Technical Bulletin. Please contact Promega Technical Services if you have questions on use of this system. E-mail techserv@promega.com.

I. Description....1

II. Product Components....4

III. Creating a Ribonuclease-Free Environment....5

IV. RNA Isolation Procedure....5
- A. Preparation of Materials....7
- B. Sample Preparation....7
- C. RNA Extraction....8
- D. RNA Precipitation and Resuspension....9
- E. RNA Wash....10

V. Determination of RNA Yield and Quality....10
- A. Yield and Purity....10
- B. Integrity....12
- C. Applications and Comparisons to Alternative Protocols....12

VI. Troubleshooting....15

VII. Appendix....16
- A. DNase Treatment of Total RNA Samples (optional)....16
- B. RT-PCR....17
- C. Composition of Buffers and Solutions....18
- D. Related Products....19
- E. References....20

I. Description

The purity and integrity of RNA isolated from tissue or cultured cells are critical for its effective use in applications such as Northern blot analysis, oligo(dT) selection of poly(A)+ RNA, cDNA library construction, translation in vitro and reverse transcription PCR[(a)] (RT-PCR). RT-PCR is a powerful method to identify and quantitate tissue-specific rare mRNAs from small amounts of total RNA and mRNA. In addition, RT-PCR allows the cloning of cDNA products without the need for constructing and screening cDNA libraries. As the use of amplification as a research tool has grown, the need for methods to rapidly isolate high-quality RNA from small amounts of starting material (i.e., tissue and cultured cells) has also increased. The RNAgents® Total RNA Isolation System is designed to address these needs.

The RNAgents® Total RNA Isolation System allows multiple RNA isolations from as little as 5mg of tissue. The system includes combined guanidine thiocyanate crystals and the CSB (Citrate/Sarcosine/β-Mercaptoethanol) Buffer in a single bottle of Denaturing Solution, provided ready to use. Furthermore, the Phenol:Chloroform:Isoamyl Alcohol Solution, provided with the system, has been optimized to remove contaminating chromosomal DNA from samples. The elimination of chromosomal DNA, a major advantage of this improved system, is an important consideration when the downstream application is amplification and analysis of RNA transcripts.

The successful isolation of intact RNA requires four essential steps: i) effective disruption of cells or tissue; ii) denaturation of nucleoprotein complexes; iii) inactivation of endogenous ribonuclease (RNase) activity; and iv) removal of contaminating DNA and proteins. Most important is the immediate inactivation of endogenous RNase activity that is released from membrane-bound organelles upon cell disruption. The RNAgents® Total RNA Isolation System uses two potent inhibitors of RNase, guanidine thiocyanate and β-mercaptoethanol (1). In addition, all procedures are performed on ice to significantly slow the rate of RNA degradation (2). Guanidine thiocyanate with N-lauryl sarcosine also acts to disrupt nucleoprotein complexes, allowing RNA to be released into solution free of protein. Contaminants are separated by acid extraction using Phenol:Chloroform:Isoamyl Alcohol (3). DNA and proteins partition into the organic phase; the RNA partitions selectively into the aqueous phase. The RNA is recovered from the aqueous phase by precipitation with isopropanol. The procedure is easy to perform with large or small quantities of tissue or cultured cells, and it can be used to process multiple samples. The use of organic solvent extraction eliminates the need for ethanol and lithium chloride (LiCl) precipitations, cesium chloride gradients and ultracentrifugation. The use of LiCl can result in the loss of RNA smaller than 5.8S (4), and carryover of lithium salts can inhibit subsequent cDNA synthesis reactions (5).

An alternative, shorter protocol is provided for purifying RNA for use in RT-PCR. This protocol, which can be completed in 90 minutes or less (Figure 1), includes a shorter isopropanol precipitation and omits a second solubilization in the Denaturing Solution and the subsequent precipitation in isopropanol. This procedure results in RNA yields similar to the standard protocol (see Table 2). For all applications other than RT-PCR, RNA should be isolated using the standard protocol.

Note: If you are interested in using RNA purified with the RNAgents® Total RNA Isolation System for RT-PCR, please see Section VII.B for a list of references for this application.

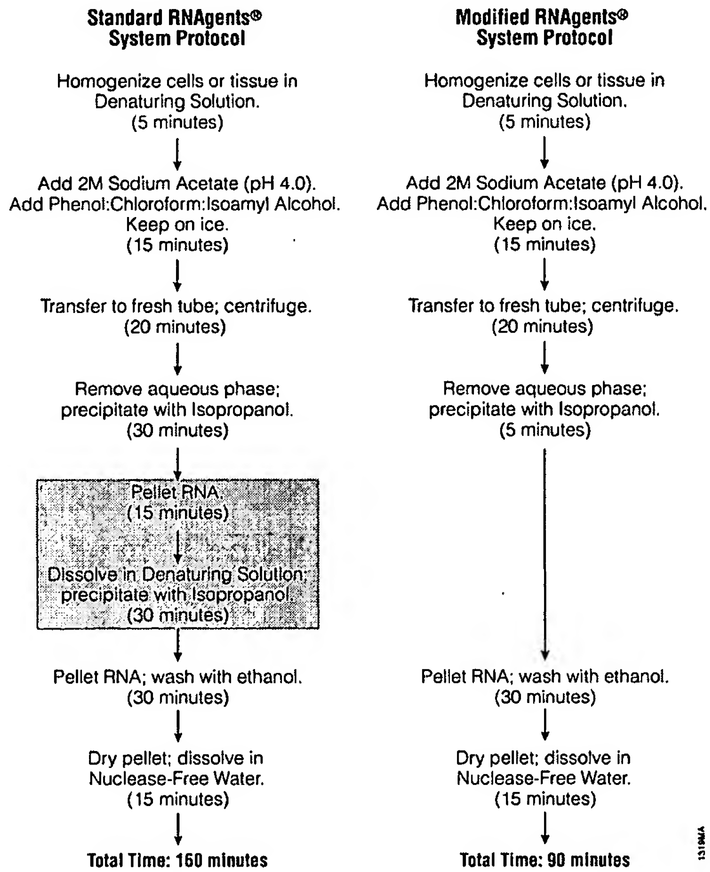


Figure 1. Summary of the standard and modified RNAgents® Total RNA Isolation System protocols. The modified RNAgents® System protocol is suggested for RT-PCR; the standard RNAgents® System protocol is recommended for all other applications.

II. Product Components

Product	Cat.#
RNAgents® Total RNA Isolation System	Z5110

For Laboratory Use. Each system contains sufficient reagents for isolation of total RNA from up to 6g of tissue or 6×10^8 cells and can be scaled proportionally as needed.

Includes:

- 120ml RNAgents® Denaturing Solution
- 10ml 2M Sodium Acetate (pH 4.0)
- 100ml Phenol:Chloroform:Isoamyl Alcohol (99:24:1, pH 4.7)
- 100ml Isopropanol
- 25ml Nuclease-Free Water
- 1 Protocol

Storage Conditions: Store the Phenol:Chloroform:Isoamyl Alcohol at 4°C. Store the remaining components at 4–25°C. All components are stable for six months from the date of purchase unless otherwise mentioned on the product label.

Note: N-lauryl sarcosine, present in the Denaturing Solution, may precipitate. If this occurs, redissolve by warming to 37°C just before use.

Caution: Guanidine thiocyanate is a potent chaotropic agent and irritant. Phenol is poisonous and can cause severe burns. Wear gloves and safety glasses when handling these reagents. If phenol contacts the skin, rinse the area immediately with large quantities of water and seek medical attention. **Do not rinse with ethanol!** For additional precautions, refer to the Material Safety Data Sheets (MSDS) available on the Internet at: www.promega.com/msds/

Available Separately

Product	Size	Cat.#
RNAgents® Denaturing Solution	120ml	Z5651

For Laboratory Use.

When using RNAgents® Denaturing Solution (Cat.# Z5651) to isolate total RNA, the user must supply phenol:chloroform:isoamyl alcohol [Sigma Cat.# P1944 phenol:phloroform 5:1 for molecular biology (125:24:1 mixture of phenol, chloroform and isoamyl alcohol)] and 2M sodium acetate.

III. Creating a Ribonuclease-Free Environment

Ribonucleases are difficult to inactivate. Therefore, care should be taken to avoid inadvertently introducing RNase activity into the isolation procedure. The following precautions will help to prevent accidental contamination of samples.

The more common sources of RNase contamination are the user's hands, and bacteria and molds that may be present on airborne dust particles. Therefore, wear gloves at all times and use proper microbiological sterile technique when handling the reagents.

We recommend using sterile disposable plasticware for handling RNA. These materials are generally RNase-free and thus do not require pretreatment to inactivate RNase.

Treat nondisposable glassware and plasticware before use to ensure that it is RNase-free. Bake glassware at 200°C overnight. Thoroughly rinse plasticware with 0.1N NaOH, 1mM EDTA and then with Nuclease-Free Water. **Note:** COREX® tubes should be rendered RNase-free by treatment with 0.1% diethyl pyrocarbonate (DEPC) instead of by baking. Baking will increase the failure rate of this type of tube during centrifugation. Alternatively, commercially available products, such as RNase AWAY® (Molecular Bio-Products), may be used to remove contaminating RNases.

Use RNase-free materials for weighing chemicals. Solutions supplied by the user should be treated by the addition of DEPC to 0.1% overnight at room temperature and then autoclaved for 30 minutes to remove any traces of DEPC. **Note:** Tris buffers cannot be treated with DEPC. Prepare Tris buffers by using a container of Tris designated only for RNA isolations and DEPC-treated water that has been autoclaved.

IV. RNA Isolation Procedure

A variety of RNA isolation protocols from samples of plant and animal tissues or from cultured cells are provided. Please identify and read the subsection that applies to the source of RNA you are using. Many of the procedures require that the solutions be chilled on ice prior to use. Section IV.A outlines the steps for materials preparation. Table 1 lists the appropriate size tube for the centrifugation steps and the volumes of each reagent required (based on the mass of starting material) for the extraction procedures.

For homogenizing a large mass of starting material (>75–1,000mg tissue or $>7.5 \times 10^6$–1×10^8 cells), use 50ml thick-walled polypropylene centrifuge tubes. For homogenizing a small mass of starting material (5–75mg tissue or 5×10^5–7.5×10^6 cells), use 2ml screw-cap polypropylene tubes. Ensure that either type of tube is free of RNase contamination.

Promega

Materials to Be Supplied by the User
(Solution compositions are provided in Section VII.C.)

- 37°C water bath or oven
- tissue homogenizer (e.g., Brinkmann, Omni Micro or Dounce glass-Teflon®)
- 50ml thick-walled polypropylene centrifuge tubes, DEPC-treated (e.g., Sorvall® Cat.# 03530 and 03535)
- 50ml polypropylene screw-cap tubes (e.g., Corning Cat.# 430828)
- 2ml screw-cap polypropylene tubes, sterile (e.g., Sarstedt Cat.# 72.693.005)
- ice-cold ethanol, 75%, RNase-free
- 10X PBS buffer, sterile
- DEPC
- refrigerated centrifuge

Table 1. Tube Size and Volumes of Reagents to be Used for Varying Tissue Mass and Cell Number.

Recommended Tube Size (minimum)	Tissue	Cultured Cells	Denaturing Solution	2M Sodium Acetate	Phenol: Chloroform: IAA	Denaturing Solution*
30ml	1,000mg	1.0×10^8	12ml	1,200µl	12ml	5ml
30ml	750mg	7.5×10^7	9ml	900µl	9ml	
15ml	500mg	5.0×10^7	6ml	600µl	6ml	
15ml	250mg	2.5×10^7	3ml	300µl	3ml	2.5ml
15ml	100mg	1.0×10^7	1.2ml	120µl	1.2ml	
2ml	75mg	7.5×10^6	900µl	90µl	900µl	
1.5ml	50mg	5.0×10^6	600µl	60µl	600µl	500µl
1.5ml	25mg	2.5×10^6	300µl	30µl	300µl	
0.5ml	10mg	1.0×10^6	120µl	12µl	120µl	100µl
0.5ml	5mg	5.0×10^5	60µl	6µl	60µl	

Abbreviation: IAA = Isoamyl Alcohol.

*Refers to the second resuspension with Denaturing Solution.

Note: Samples of 75mg or less, or 7.5×10^6 cells or fewer, can be processed in microcentrifuge tubes. For tissue mass or cell numbers not listed in Table 1, use the next highest listed volume or proportionally scale the solutions to the sample size.

Do not exceed 1g tissue or 1×10^8 cells per 12ml (or 1µg per 5µl) of Denaturing Solution.

A. **Preparation of Materials**

1. Pretreat the 50ml thick-walled polypropylene centrifuge tubes in 0.1% diethyl pyrocarbonate (DEPC) for one hour at room temperature and then autoclave the tubes for 30 minutes to destroy any residual DEPC. Alternatively, treat the tubes with an RNase-removing agent such as RNase AWAY®.

2. Allow the provided Phenol:Chloroform:Isoamyl Alcohol to warm to room temperature for 15 minutes. Ensure that the phases are separated.

3. Before use, chill the Denaturing Solution on ice. If needed, prepare and chill 1X PBS buffer before use.

B. **Sample Preparation**

Samples to be extracted should be as fresh as possible to obtain optimal performance from this system. Otherwise, freeze the samples **immediately** in liquid nitrogen and store at -70°C for future use. The volumes of reagents may be adjusted proportionally for different amounts of starting material (Table 1). Samples homogenized in Denaturing Solution may be stored indefinitely at -70°C. For valuable samples, we suggest that a portion of each sample be reserved at -70°C (in the event that loss of a sample occurs during the procedure).

Animal Tissue

1. Dispense the recommended volume of Denaturing Solution into a sterile tube and chill on ice for five minutes.

   Note: When working with >75–1,000mg of tissue, we recommend using a 50ml, polypropylene screw-cap tube. These tubes will handle most standard size tissue disruption probes. For 5–75mg tissue samples, use a 2ml screw-cap tube. However, a tissue disrupter (such as the Omni Micro Homogenizer) equipped with a microprobe will be needed.

2. Place the fresh or frozen tissue sample into the Denaturing Solution and disrupt the tissue with a homogenizer on high setting for 15–30 seconds. Ensure that no fragments of tissue or clumps of cells are visible.

   Alternatively, if a mechanical homogenizer is not available, mince the tissue with a clean razor blade and disrupt with a Dounce glass-Teflon® homogenizer. Process the samples as quickly as possible and proceed to Section IV.C.

Suspension Culture Cells

Place cells in the appropriately sized tube (described in Table 1) and pellet the cells by centrifugation at 300 × *g* for 5 minutes at 4°C. Wash the cell pellet with 25ml of ice-cold, sterile 1X PBS and repeat the centrifugation. Decant the supernatant, add chilled Denaturing Solution (refer to Table 1 for the appropriate volume) and vortex, or homogenize as described in Step 2 of Section IV.B (Animal Tissue). Proceed to Section IV.C.

Adherent Cells

The following volumes are designed for processing 1×10^8 cells. If fewer cells are to be processed, refer to Table 1 for appropriate volumes and tube sizes. Calculate the total number of flasks needed to provide approximately 1×10^8 cells. Decant the culture medium from each flask and wash the cells with ice-cold, sterile 1X PBS.

1. Add 8ml of chilled Denaturing Solution to one of the flasks and rock by hand until the cells lyse. The solution will become very viscous.
2. Carefully transfer the cell slurry from the first flask to the next flask, continuing the lysis procedure of Step 1 until all the cells of each flask have been lysed.
3. Add 4ml of Denaturing Solution to the first flask. Rotate the solution thoroughly over the bottom of the flask to remove any remaining cells. Transfer this solution to each subsequent flask, as in Steps 1 and 2, to collect the remaining cells and lysate.

   Note: If any cells remain, use an additional 5–10ml of Denaturing Solution to wash the flasks. In this case, proportionately increase the amounts of other reagents to be added subsequently.
4. Transfer the 12ml (or more) of lysed cells to a sterile 50ml centrifuge tube and vortex, or homogenize the cells as described in Step 2 of Section IV.B (Animal Tissue). Proceed to Section IV.C.

Plant Tissue

1. Dispense the recommended volume of Denaturing Solution (see Table 1) into a sterile 50ml conical cell culture tube and chill on ice for five minutes.
2. Freeze the freshly harvested tissue in liquid nitrogen.
3. Grind the tissue under liquid nitrogen using a ceramic mortar and pestle.
4. Allow the liquid nitrogen to evaporate, transfer the ground plant tissue to a sterile 50ml conical cell culture tube and homogenize as described in Step 2 of Section IV.B (Animal Tissue). Proceed to Section IV.C.

C. RNA Extraction

Note: When using RNAgents® Denaturing Solution (Cat.# Z5651) to isolate total RNA, the user must supply phenol:chloroform:isoamyl alcohol [Sigma Cat.# P1944 phenol:chloroform 5:1 for molecular biology (125:24:1 mixture of phenol, chloroform and isoamyl alcohol)] and 2M sodium acetate.

1. Add the recommended amount of 2M Sodium Acetate (pH 4.0) from Table 1 and mix thoroughly by inverting the tube 4–5 times.
2. Add the recommended amount of Phenol:Chloroform:Isoamyl Alcohol (Table 1) to the tube, being careful to remove only from the lower organic phase. Cap the tube, carefully mix by inversion 3–5 times and then shake vigorously for 10 seconds. Chill on ice for 15 minutes.

3. Transfer this mixture to the appropriate size DEPC-treated tube or sterile microcentrifuge tube and centrifuge at 10,000 × *g* for 20 minutes at 4°C. Use either a fixed-angle or swinging bucket rotor.

4. Carefully remove the **top aqueous phase** that will contain the RNA and transfer it to a fresh DEPC-treated tube. DNA and proteins will remain in the organic phase and at the interface. Be careful to avoid taking material from the interface, which contains genomic DNA.

D. RNA Precipitation and Resuspension

The following procedure includes all the steps for the isolation of total RNA for use in any downstream application. If the RNA is to be used for RT-PCR, Steps 3 and 4 may be omitted; **the omission of Steps 3 and 4 constitute the modified protocol for use of the RNA sample in RT-PCR** (see Figure 1). We have observed no difference in the purity or performance of RNA isolated from mouse liver, as measured by A_{260}/A_{280}, Northern blotting or RT-PCR, between the two protocols.

1. Add an equal volume of the provided Isopropanol to the aqueous phase and incubate the sample at -20°C for at least 5 minutes to precipitate the RNA.

   Note: To obtain the maximum yield from tissue samples that contain relatively low amounts of RNA, we recommend longer (30 minute) precipitations.

2. Pellet the RNA by centrifugation at 10,000 × *g* for 10 minutes at 4°C.

3. Resuspend the RNA pellet in the recommended volume of Denaturing Solution (Table 1) and vortex until the RNA is dissolved. In some instances, heating to 65°C may be required to dissolve the pellet. Heat for as short a time as possible.

4. Add an equal volume of Isopropanol and precipitate the RNA as described in Steps 1 and 2 above. Pellet the RNA by centrifugation at 10,000 × *g* for 10 minutes at 4°C. Proceed to Section IV.E.

E. RNA Wash

1. Wash the pellet by adding a minimum of 1ml of ice-cold 75% ethanol for small samples (in a microcentrifuge tube) or 10ml of 75% ethanol for large samples. Break up the pellet with a sterile, RNase-free pipette tip or glass rod and centrifuge at 10,000 × *g* for 10 minutes at 4°C.
2. Air-dry the pellet in an RNase-free environment or in a vacuum desiccator for 5–20 minutes, depending on the size of the pellet. **Do not overdry the pellet.** Desiccated RNA is extremely difficult to resuspend.
3. Use the average RNA yields given in Tables 2 and 3 to estimate the volume needed to dissolve the RNA at a convenient concentration for the intended application. Dissolve the RNA in the appropriate volume of the provided Nuclease-Free Water or in RNase-free TE buffer (Section VII.C), and store at -20°C.

   For **long-term storage**, we recommend ethanol precipitation of the RNA; add 2M Sodium Acetate (pH 4.0) to a concentration of 0.25M and 2.5 volumes of 100% ethanol. Mix by inversion, and store at -70°C.

V. Determination of RNA Yield and Quality

A. Yield and Purity

The RNAgents® Total RNA Isolation System can be used to generate large amounts of intact RNA from a variety of tissue and cell sources. The yield of total RNA may be determined spectrophotometrically at 260nm, where 1 absorbance unit (A_{260}) = 40µg of single-stranded RNA/ml. The purity may be estimated, also by spectrophotometry, from the relative absorbances at 230, 260 and 280nm (i.e., A_{260}/A_{280}).

Table 2 lists representative values for RNA yield and purity, by ratios for A_{260}/A_{280} and A_{260}/A_{230}, from mouse liver. Figure 2 shows the linearity of total RNA yield isolated from 5–1,000mg of mouse liver.

Table 2. Yield of RNA per Gram of Mouse Liver Tissue.

System/Protocol	Average Yield	Average A_{260}/A_{280}	Average A_{260}/A_{230}
RNAgents® System, Standard Protocol	6.6mg	1.99	1.64
RNAgents® System, Modified Protocol	6.8mg	1.97	1.68

Note: Averages were from three independent determinations.

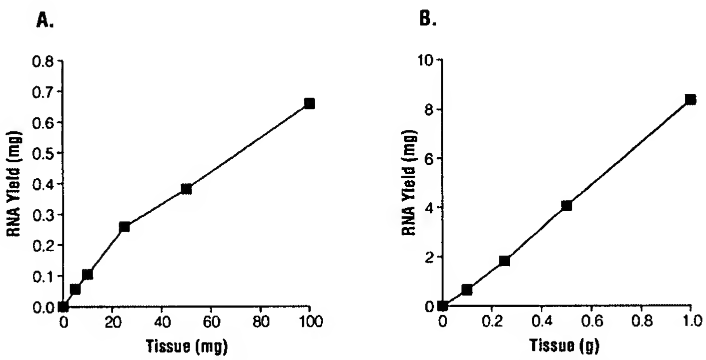


Figure 2. Yield of total RNA versus tissue amount using the RNAgents® System. The yield of total RNA from mouse liver was determined from increasing amounts of tissue using the Modified RNAgents® System Protocol as outlined in Figure 1. **Panel A.** 5–100mg tissue samples. **Panel B.** 100–1,000mg tissue samples.

RNA isolated with the RNAgents® System is substantially free of DNA and contaminating protein and may be used directly for any of the applications discussed in Section I. Pure RNA will exhibit an A_{260}/A_{280} ratio of 2.0. However, it should be noted that, due to the variations between individual starting materials and in performing the procedure, the range of expected RNA by A_{260}/A_{280} ratio will be 1.7–2.0. If the RNA exhibits a ratio <1.7, refer to Section VI for possible causes and suggestions on improving the purity of the RNA. **Also, if the presence of trace amounts of genomic DNA will be a problem, a protocol is provided in Section VII.A to digest any DNA while preserving the integrity of the RNA.** Table 3 lists representative yields and A_{260}/A_{280} ratios of total RNA isolated from a number of different cell and tissue sources using the RNAgents® System with the optional steps (Section IV.D, Steps 3 and 4). Using this protocol, the RNA will usually exhibit an A_{260}/A_{230} ratio of 1.5–2. Ratios lower than this generally indicate carryover contamination by guanidine thiocyanate during the precipitation steps. Refer to Sections VI and VII.A for suggestions on improving the purity of the isolated RNA.

Table 3. Yields and A_{260}/A_{280} Ratios of Total RNA Isolated from Tissues and Cells Using the Standard Protocol.

Tissue Source	Yield of Total RNA	A_{260}/A_{280}
HeLa cells	1.6mg RNA/10^8 cells	1.85
Human WBC	1.3mg RNA/10^8 cells	1.72
Mouse intestine	2.3mg RNA/g tissue	1.75
Mouse spleen	8.3mg RNA/g tissue	1.67
Mouse lung	1.9mg RNA/g tissue	1.75
Mouse liver	6.6mg RNA/g tissue	1.99
Mouse kidney	3.1mg RNA/g tissue	1.70

Promega

B. Integrity

Determine the integrity of the purified RNA by denaturing agarose gel electrophoresis. Several methods are suitable for this purpose, using either formaldehyde (6,7) or glyoxal (7,8) as the denaturing agent. The ratio of 28S to 18S eukaryotic ribosomal RNAs should be approximately 2:1, by ethidium bromide staining, indicating that no gross degradation of RNA has occurred (see Figure 3). In RNA samples that have been degraded, this ratio will be reversed since the 28S ribosomal RNA characteristically is degraded to an 18S-like species. Refer to Sections III and VI for suggestions on avoiding RNA degradation.

C. Applications and Comparisons to Alternative Protocols

RNA samples isolated from mouse liver were evaluated by Northern analysis since the integrity of RNA is of critical importance (Figure 4). The RNA, isolated using the standard protocol, was found to be intact. Using a mouse-specific α-1-antitrypsin probe, message was detected in as little as 0.75μg of total RNA.

The sensitivity of detection achieved with the modified protocol, as determined by 35 rounds of cDNA amplification of the rare cytokine message IL-1β, was similar to the standard protocol and to the competitor's protocol (Figure 5). In this experiment, shortening the RNA isolation protocol does not affect the integrity of the RNA or the sensitivity of detection by RT-PCR (9).

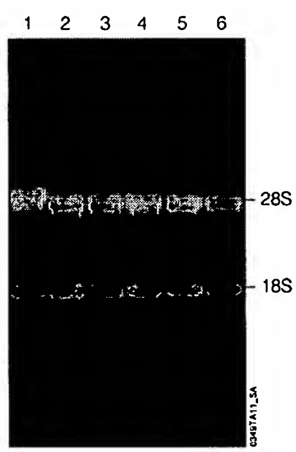


Figure 3. Analysis of RNA isolated from various tissues with the RNAgents® System. RNA was isolated from the following sources: HeLa cells (lane 1), mouse intestine (lane 2), mouse spleen (lane 3), mouse lung (lane 4), mouse kidney (lane 5) and mouse liver (lane 6). Five micrograms of each sample was resolved on a 1% denaturing agarose gel (2.2M formaldehyde).

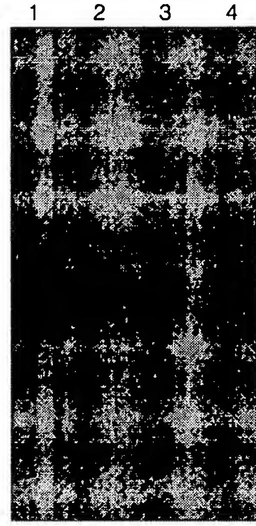


Figure 4. Detection of α-1-antitrypsin in Northern blot of mouse liver RNA using the RNAgents® Total RNA Isolation System. RNA was isolated using the standard protocol for the RNAgents® System and loaded on a 1% MOPS formaldehyde-agarose gel as follows: 6μg (lane 1), 3μg (lane 2), 1.5μg (lane 3), and 0.75μg (lane 4). RNA samples were resolved by electrophoresis, stained with ethidium bromide and transferred to a neutral nylon membrane in 20X SSC for 18 hours. The blot was washed in 2X SSC for 5 minutes, blotted dry, and the RNA was crosslinked by UV light using the Stratalinker® instrument on auto setting. The membrane then was baked under vacuum at 80°C for 30 minutes. The membrane was prehybridized (in 5X SSC, 10% dextran sulfate, 40% deionized formamide and 0.5% SDS) for 1 hour at 42°C and hybridized overnight (17 hours) at 42°C with a random-primed radiolabeled, mouse specific α-1-antitrypsin probe (5ng/ml) in prehybridization buffer. Following stringency washes of 5X SSC, 1% SDS and 0.1X SSC, 0.1% SDS for 15 minutes at 65°C each, the blot was exposed to X-OMAT® film for 3.5 hours at 25°C without an intensifying screen.

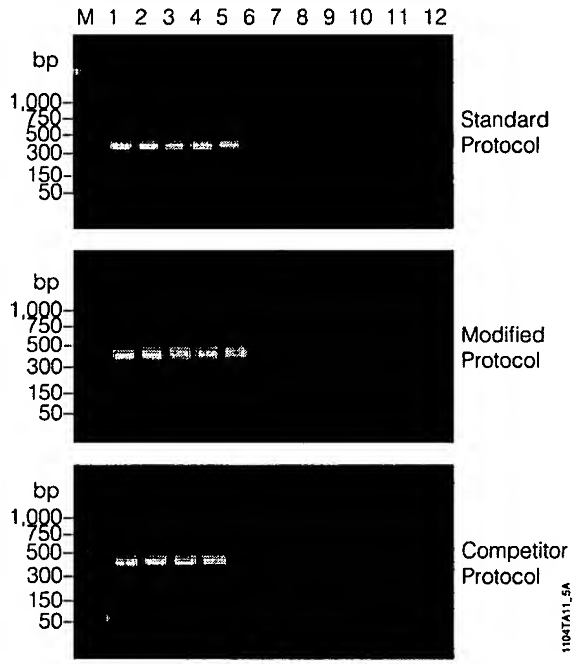


Figure 5. Amplification analysis of cDNA prepared from total RNA. Total RNA was prepared from mouse liver using the standard and modified protocols of the RNAgents® System and the protocol of another commercially available RNA extraction kit. The RNA was diluted in a three-step series of dilutions and reverse transcribed using AMV Reverse Transcriptase (Cat.# M5101), Recombinant RNasin® Ribonuclease Inhibitor (Cat.# N2511) and an Oligo(dT)$_{15}$ Primer (Cat.# C1101). Amplification efficiency of each reaction was compared using primers for the rare cytokine message IL-1β. Amplification was performed using the protocols provided with *Taq* DNA Polymerase. One-sixth (5µl) of each cDNA sample was amplified in a 50µl reaction containing primers at a concentration of 1µM each (primer sequences and amplification conditions were provided by Dr. A.L. Oaklander, Johns Hopkins University). Two-fifths (20µl) of each amplification reaction was analyzed by 2% agarose gel electrophoresis and ethidium bromide staining. The lanes represent PCR products from decreasing amounts of RNA template: 1µg (lane 1), 333ng (lane 2), 111ng (lane 3), 37ng (lane 4), 12ng (lane 5), 4ng (lane 6), 1.4ng (lane 7), 457pg (lane 8), 152pg (lane 9), 51pg (lane 10), 17pg (lane 11), and no RNA (lane 12). Lane M contains PCR Markers (Cat.# G3161).

VI. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: www.promega.com. E-mail: techserv@promega.com

Symptoms	Causes and Comments
Low A_{260}/A_{280} ratios	Protein contamination. Several methods may be used for further removal of contaminating protein from RNA. The most expedient method is to perform an additional phenol:chloroform extraction on the purified RNA. Repeat the procedures in Sections IV.C–IV.E. This additional organic solvent extraction should yield higher A_{260}/A_{280} ratios. However, loss of RNA (as much as 40%) should be expected.
Low A_{260}/A_{230} ratios	Guanidine thiocyanate contamination. Precipitate the RNA by adding 0.1 volume of 2M Sodium Acetate (pH 4.0) and an equal volume of Isopropanol. Incubate at –20°C for 30 minutes, and collect the RNA by centrifugation at 10,000 × *g* for 15 minutes at 4°C. Resuspend the RNA in 1mM EDTA (RNase-free) or Nuclease-Free Water, and precipitate the RNA as described above. Add a minimum of 1ml or 15ml of 75% ethanol for samples in small or large tubes, respectively. Break up the pellet, either by vortexing or with a sterile RNase-free glass rod, and centrifuge at 10,000 × *g* for 15 minutes. Remove the ethanol, dry the pellet in a vacuum desiccator for 15–20 minutes, and resuspend in Nuclease-Free Water.
Presence of high molecular weight nucleic acids (detected at the well in ethidium bromide-stained gels or by RT-PCR)	DNA contamination. See Section VII.A on DNase treatment of RNA samples.
RNA degradation	RNase introduced by handling, or RNase not entirely inactivated. Use DEPC-treated glassware and solutions. A common misconception is that guanidine thiocyanate irreversibly denatures RNase. If a small amount of denatured RNase is present at the end of the procedure, it may renature when denaturing agents are removed, then degrade the sample. This is most likely with samples that contain very high amounts of endogenous RNase, such as rat pancreas.

VII. Appendix

A. DNase Treatment of Total RNA Samples (optional)

If the presence of trace amounts of contaminating genomic DNA will be problematic for a particular application, the following protocol may be used to remove DNA from the total RNA preparation.

Materials to Be Supplied by the User
(Solution compositions are provided in Section VII.C.)

- 37°C water bath
- RQ1 RNase-Free DNase (Cat.# M6101)
- RQ1 DNase buffer, 10X (RNase-free)
- Recombinant RNasin® Ribonuclease Inhibitor, 20–40u/µl (Cat.# N2511)
- ethanol, 70 and 100%, nuclease-free
- chloroform:isoamyl alcohol (24:1)
- RNase-free TE buffer (pH 7.4)
- DTT, 100mM (Cat.# P1171)
- 3M sodium acetate (nuclease-free; no pH adjustment)

1. Digest the RNA sample with RQ1 RNase-Free DNase by setting up the following reaction. Add the components in the order that they are listed.

Total RNA sample (1–2µg/µl; in Nuclease-Free Water or TE buffer)	50µl
Nuclease-Free Water	Xµl
RQ1 DNase buffer, 10X (RNase-free)	10µl
DTT, 100mM	10µl
RQ1 RNase-Free DNase	2 units
Recombinant RNasin® Ribonuclease Inhibitor	100 units
Final volume of	100µl

2. Incubate at 37°C for 15 minutes.

3. Extract the RNA with an equal volume (100µl) of the Phenol:Chloroform: Isoamyl Alcohol (99:24:1, pH 4.7), vortex for 1 minute and spin in a microcentrifuge at 12,000 × g for 2 minutes. Transfer the upper, aqueous phase to a new tube and perform a second extraction using chloroform:isoamyl Alcohol (24:1).

   Note: The treated samples must be extracted with Phenol:Chloroform:-Isoamyl Alcohol to remove the RQ1 RNase-Free DNase.

4. Transfer the upper, aqueous phase to a new tube, and ethanol precipitate the RNA by adding sodium acetate to 0.3M and 2.5 volumes of 100% ethanol. Mix and place at –20°C for 15–30 minutes, then spin in a microcentrifuge at 12,000 × *g* for 5 minutes. Wash the RNA pellet with 1ml of RNase-free 70% ethanol and dry, not completely, under vacuum. Dissolve the RNA in Nuclease-Free Water (provided) or in RNase-free TE buffer (Section VII.C). Store at –70°C.

5. Determine the RNA concentration as described in Section V. If desired, analyze an aliquot of the RNA by gel electrophoresis (7).

B. RT-PCR

The online Protocol and Applications Guide, Chapter 1, covers general considerations for RT-PCR and can be found on Promega's web site at: **www.promega.com/paguide/**. References 10 and 11 offer more in-depth information on PCR. Reference 12 presents a comparison of different reverse transcriptases used in RT-PCR.

Promega offers the Access RT-PCR System (Cat.# A1280 and A1250) and the Access RT-PCR Introductory System (Cat.# A1260) for the generation of PCR products from total or messenger RNA. The Access RT-PCR Systems use AMV (Avian Myeloblastosis Virus) RT for first-strand cDNA synthesis and *Tfl* (*Thermus flavus*) DNA Polymerase for second-strand cDNA synthesis and DNA amplification (13). Articles that describe the Access RT-PCR System and the RNAgents® Total RNA Isolation System can be found in references 14 and 9, respectively.

Alternatively, the ImProm-II™ Reverse Transcription System (Cat.# A3800) can be used for first-strand cDNA synthesis, then coupled with a thermostable polymerase such as *Taq* DNA Polymerase (Cat.# M1661) for PCR amplification.

C. Composition of Buffers and Solutions

Note: As indicated in Section III, solutions supplied by the user should be treated by the addition of DEPC (except for Tris-based solutions and PBS buffer) to 0.1% overnight at room temperature, then autoclaved for 30 minutes to remove any traces of DEPC. Tris buffers cannot be treated with DEPC. Prepare Tris buffers by using a container of Tris designated only for RNA isolations. Use RNase-free materials for weighing chemicals, and use DEPC-treated water for solutions.

Denaturing Solution (provided)

26mM	sodium citrate (pH 4.0)
0.5%	N-lauryl sarcosine
0.125M	β-mercaptoethanol
4M	guanidine thiocyanate

PBS buffer, 10X

11.5g	Na_2HPO_4
2g	KH_2PO_4
80g	NaCl
2g	KCl

Dissolve in 1L of sterile, deionized water and autoclave. The pH of 1X PBS should be 7.4.

RQ1 DNase buffer, 10X (RNase-free)

400mM	Tris-HCl (pH 7.9)
100mM	NaCl
60mM	$MgCl_2$
100mM	$CaCl_2$

TE buffer (pH 7.4, RNase-free)

10mM	Tris-HCl (pH 7.4)
1mM	EDTA (pH 8.0)

Note: Tris-containing solutions cannot be treated with DEPC. Prepare in Nuclease-Free Water and reserve a fresh stock of TE buffer for use in RNA procedures only. Alternatively, use 0.1% DEPC-treated water that has been autoclaved before adding Tris.

2M Sodium Acetate (pH 4.0)
Add 164.1g of sodium acetate, anhydrous (F.W. 82.03) to 300ml Nuclease-Free Water. Bring to pH 4.0 ± 0.2 using glacial acetic acid. Bring to a final volume of 1 liter with nuclease-free water.

Phenol:Chloroform:Isoamyl alcohol
Customers using the RNAgents® Denaturing Solution (Cat.# Z5651) as a standalone item will need to provide their own phenol:-chloroform:isoamyl alcohol solution [Sigma Cat.# P1944 (phenol:chloroform 5:1 for molecular biology, 125:24:1 mixture of phenol, chloroform and isoamyl alcohol).

ethanol, 70% and 75%
Dilute 95% or 100% ethanol to 70% and 75% (v/v) with DEPC-treated water.

D. Related Products

Total RNA Isolation Systems

Product	Size	Cat.#
SV Total RNA Isolation System*	50 preps	Z3100
	250 preps	Z3105
SV Total RNA Isolation System, Trial Size*	10 preps	Z3101

Product	Size	Cat.#
SV 96 Total RNA Isolation System*	1 × 96 each	Z3500
	5 × 96 each	Z3505

Product	Size	Cat.#
Miniprep Vacuum Adapters	20 each	A1331
SV Red Blood Cell Lysis Solution*	200ml	Z3141
SV RNA Lysis Buffer*	50ml	Z3051

*For Laboratory Use.

RNA Isolation from Total RNA

Product	Cat.#
PolyATtract® mRNA Isolation System I	Z5210

For Laboratory Use. Each system contains sufficient reagents for three separate mRNA isolations, each from 1–5mg total RNA.

Product	Cat.#
PolyATtract® mRNA Isolation System III	Z5300

For Laboratory Use. Each system contains sufficient reagents for fifteen separate mRNA isolations, each from 100–1,000μg total RNA.

mRNA Isolation Directly from Biological Samples

Product	Cat.#
PolyATtract® System 1000	Z5420

For Laboratory Use. Each system contains sufficient reagents to isolate mRNA from up to 2g of tissue or for isolations from up to 4×10^8 tissue culture cells.

Product	Cat.#
PolyATtract® System 1000 Magnetic Separation Stand	Z5410

RNA Markers

Product	Size	Cat.#
RNA Markers	50μl	G3191

For Laboratory Use.

Amplification-Related Products

Please see our product catalog, available upon request from Promega, or visit our web site at: www.promega.com/catalog/ for a complete listing of our RNA isolation and amplification products.

E. References

1. Chirgwin, J.M. *et al.* (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–9.
2. Han, J.H. *et al.* (1987) Isolation of full-length putative rat lysophospholipase cDNA using improved methods for mRNA isolation and cDNA cloning. *Biochemistry* **26**, 1617–25.
3. Perry, R.P. *et al.* (1972) On the lability of poly(A) sequences during extraction of messenger RNA from polyribosomes. *Biochim. Biophys. Acta* **262**, 220–6.
4. Stern, D.B. and Newton, K.J. (1986) Isolation of plant mitochondrial RNA. *Meth. Enzymol.* **118**, 488–96.
5. Wallace, D.M. (1987) Precipitation of nucleic acids. *Meth. Enzymol.* **152**, 41–8.
6. Lehrach, H. *et al.* (1977) RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry* **16**, 4743–51.
7. Ausubel, F.M. *et al.*, eds. (1993) In: *Current Protocols in Molecular Biology*, Greene Publishing Associates and John Wiley and Sons, New York, NY.
8. McMaster, G.K. and Carmichael, G.G. (1977) Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl. Acad. Sci. USA* **74**, 4835–8.
9. Brisco, P., Andersen, B. and Smith, C. (1996) A rapid protocol for the isolation of total RNA suitable for RT-PCR. *Promega Notes* **55**, 10–15.
10. Ferre, F. (1992) In: *PCR Methods and Applications* **2**, 1.
11. Innis, M.A. *et al.*, eds. (1990) In: *PCR Protocols: A Guide to Methods and Applications*, Academic Press, Inc., Orlando, 21.
12. Cusi, M.G., Valassina, M. and Valensin, P.E. (1994) Comparison of M-MLV reverse transcriptase and *Tth* polymerase activity in RT-PCR of samples with low virus burden. *BioTechniques* **17**, 1034–6.
13. *Access RT-PCR System Technical Bulletin* #TB220, Promega Corporation.
14. Miller, K. and Storts, D. (1995) PCR ACCESS! A sensitive single-tube, two-enzyme system for RT-PCR. *Promega Notes* **53**, 2-5

[a]The PCR process is covered by patents issued and applicable in certain countries*. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process.
*In the U.S., effective March 29, 2005, U.S. Pat. Nos. 4,683,195, 4,965,188 and 4,683,202 will expire. In Europe, effective March 28, 2006, European Pat. Nos. 201,184 and 200,362 will expire.